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Nanosecond Pulsed Electric Fields Induce a Mitochondria-Independent Apoptosis in B16F10 Melanoma Cells *In Vitro*

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by

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ABSTRACT

NANOSECOND PULSED ELECTRIC FIELDS INDUCE A MITOCHONDRIA-INDEPENDENT APOPTOSIS IN B16F10 MELANOMA CELLS *IN VITRO*

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Nanosecond pulsed electric fields (nsPEFs) are ultra-short pulses that induce direct electric field and biological effects that initiate apoptosis. Here the application of ten 300ns pulses ranging in electric fields from 12kV/cm-60kV/cm was administered to determine the effects on B16F10 melanoma cells evaluated by *in vitro* studies. Initial application of nsPEFs demonstrated apoptosis induction in an electric field- and pulse number-dependent manner measured by caspase activation that correlated with decrease in cell viability 24hr post pulse. In addition caspase activity was shown to be independent of calcium mobilization though ions may play a part in other aspects of apoptosis. The use of additional apoptotic markers Annexin V and propidium iodide revealed no phosphatidylserine externalization and no direct electric field effects on membrane permeability, respectively. However, characteristic apoptotic morphological features of plasma membrane blebbing and F-actin cytoskeleton disruption were observed. In order to define apoptotic pathway, evaluation of mitochondrial function illustrated no intracellular release of mitochondrial proteins, cytochrome c, second mitochondria-derived activator of caspase (Smac), direct IAP-binding protein (DIABLO) and apoptosis inducing factor (AIF). Further examination of caspases with isozymes

specific initiators indicated electric field-dependent increases in caspases -8 and interestingly -9 as well as executioner caspases -3, -6 and -7.

NsPEFs activate signal transduction mechanisms that induce apoptosis through a mitochondria independent pathway that mimics an apparent membrane-dependent extrinsic type I apoptotic mechanism(s). Uniquely, nsPEF activate caspase -9 either via direct cleavage or an unknown mechanism without apoptosome formation in the mitochondria-dependent apoptotic pathway. Therefore nsPEFs induce apoptosis rapidly by mechanisms that are distinct and independent from those that are commonly mutant in the mitochondria pathway in melanomas and those same mechanisms frequently used by chemotherapeutic agents that result in resistant tumors. The short nsPEF treatment time (3 milliseconds) and rapid apoptosis induction (tens of minutes) is less likely to result in cancers that are resistant to nsPEF treatment. The therapeutic application of nsPEFs can provide a new and novel method to the arsenal for cancer treatment strategies.

This dissertation is dedicated to my mother, Evangeline Ford, for her unselfish sacrifice, dedication and support throughout the completion of this degree.

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BACKGROUND, SPECIFIC AIMS AND SIGNIFICANCE

BACKGROUND

The Skin

The skin is known as the largest organ in the body that accounts for approximately sixteen percent of the total body weight in adults. The skin serves as a protective barrier against physical, chemical, and biological toxic substances as well as an immunological organ that is composed of three layers, the epidermis, dermis and subcutaneous adipose tissue (Mills, 2007). The epidermis, the outermost (external) layer of skin, consists primarily of a stratified squamous keratinized epithelium comprised of five layers of keratinocytes. From the outermost to innermost epidermal layer, they are the (i) *stratum corneum*, flattened non-nucleated keratinized cells that consist of fifteen to twenty layers; (ii) *stratum lucidum*, composed of three to five layers of translucent thin flattened eosinophillic epidermal cells with no organelles and nuclei and cytoplasm composed of keratin filaments; (iii) *stratum granulosum*, composed of three to five layers of flattened polygonal cells that contain coarse basophilic granules intracellularly and protects against invasion by foreign materials and essential in the sealing effect in the skin; (iv) *stratum spinosum*, contains two to three layers of cuboidal or slightly flattened cells with central nucleus and keratin filaments in cytoplasm that holds cells together and preventing the effects of abrasion; and (v) *stratum basale (stratum germinativum)*, the lowermost epidermal layer adjacent to the dermis characterized by one to two layers of

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basophilic columnar or cuboidal cells that contain stem cells distinguished by rapid mitotic division and replenishment of epidermal cells (Junqueira and Carneiro 2005).

The multilayer dermis is the subsequent supportive connective tissue layer between the epidermis and subcutaneous adipose tissue layer. The layer is abundant in nerve supply contains cellular components, tiny blood and lymph vessels, macrophages, extravasated leukocytes, collagen fibrils, elastic fibers (responsible for skin elasticity) and epidermal derivatives that include hair follicles and sweat and sebaceous glands. Lastly, the subcutaneous adipose tissue layer, the deepest skin layer, has connective tissue that attaches the skin loosely to the underlying organs and adipose cells that differ in content depending on the location in the body and physical state of individual (Junqueira and Carneiro 2005).

Melanoma

Melanoma is a form of skin cancer that results from a disease in the pigment producing cell, melanocytes. These cells, responsible for skin color, are located in the lowermost part of the epidermis called the stratum basale (stratum germinativum). Though there are many factors that attribute to skin color, melanin and carotene content, blood vessels in the dermis and blood flow through these vessels are the main components. Melanocytes produce a dark brown pigment, eumelanin found beneath or between cells of the stratum basale and hair follicles. These cells have spherical cell bodies with long irregular projections that extend between the strata basale and spinosum. Cells are anchored to basal lamina by hemidesmosomes. The activity of tyrosinase plays an essential part in the synthesis of melanin by melanocytes. Tyrosinase activity is

initially involved in converting 3,4-dihydroxy-phenylalanine (dopa) to dopaquinone which after a series of transformations produces melanin (Junqueira and Carneiro 2005).

The maturation of melanin granules are categorized into four stages. Initially tyrosinase activity and formation of fine granular material is initiated by membrane enclosing a vesicle. Tyrosinase molecules on the protein matrix become present on the periphery of electron dense strands. The vesicle (melanosome) then deposits melanin on the protein matrix. This results in the enhancement of melanin production. Finally the melanin granule matures completely filling the vesicle. After formation, the melanin granules move toward melanocyte extension ends and are transferred from strata basale to the strata spinosum epidermal layer. The melanin granules migrate into kerotinocytes and surround the nuclei protecting it from the harmful effects of the UV radiation. The excessive amounts of melanin in kerotinocytes are removed by lysosome, which accounts for the disappearance in the upper epithelial layer in the skin. The interaction between melanocytes and kerotinocytes, rate of melanin granule formation in melanocytes, transfer to kerotinocytes and deposit of melanin granules regulate the skin color (Junqueira and Carneiro 2005).

The inability of keratinocytes to regulate melanocyte growth due to keratinocytes loss of control over melanogenesis leads to malignant transformation evident by rapid proliferation, loss of dendrite formation and melanoma-associated antigen (MAA) expression significant for metastasis and invasion (Shih et al., 1994; Hsu et al., 2000). The melanocytes enter the dermis by infiltrating through the basal lamina entering blood and lymphatic vessels migrating throughout the body (Junqueira and Carneiro 2005).

The development of melanoma is categorized into five stages. First, the common acquired nevus which is the initial formation of hyperplastic melanocytic lesions. Second, dsyplastic nevus is an abnormal mole without a well-defined border that is potentially a precursor for cutaneous melanoma. Third, radial growth phase (RGP) primary melanoma which is classified as the first malignant stage. At this point cells are restricted to the epidermis or locally invasive but do not demonstrate rapid growth or metastatic ability. Fourth, vertical growth phase (VGP) primary melanoma lesions, in which melanoma cells permeate the dermis and subcutaneous tissue with capability of entering systemic circulation. Fifth, metastasis, which is the advance stage of tumor progression (Hsu et al., 2002).

Factors That Influence Melanoma Development

Numerous factors influence the development of melanoma. The continuous and excessive exposure to sunlight over a lengthy period of time can attribute to premature aging (wrinkles) and development of basal cell, squamous cell and melanoma skin cancers. Such long term exposure from childhood to adulthood can significantly enhance the risk of melanoma development (Osterlind et al., 1988). The emission of UV radiation from the sunlight penetrates the epidermal layer of the skin that can cause deoxyribonucleic acid (DNA) damage in cells (Cifone and Fidler, 1981), immune system suppression (Noonan and De Fabo, 1985), and antioxidant inhibition (Fuch et al., 1989). UV radiation can also induce mutations in genes that regulate cell growth such as *p16*, a tumor suppressor gene important in cell cycle regulation (Ziegler et al., 1994). The loss of Langerhan cells, which are vital for initiation and maintenance of specific-T-cell

mediated responses in the skin associated with aging, is another factor linked to melanoma (Stene et al., 1988).

Melanoma Statistics

Skin cancer accounts for over 50% of all cancers. Melanoma is the least common skin cancer and accounts for approximately 3% of skin cancers that leads to a substantial amount of skin cancer deaths due to highly aggressive metastatic ability. Though non-melanoma skin cancers such as basal and squamous cell carcinomas are not considered as aggressive as melanoma, they can be potentially detrimental and cause disfigurement. Melanoma can develop at a younger age than most cancers with about 50% of individuals under age fifty-seven. In adolescents between the ages of 15 to 19, 1 in every 15,000 boys and 30,000 girls will develop melanoma. The rate of incidence is enhanced with aging being the highest among individuals in their 80's. Overall melanoma is ten times more likely to occur in Caucasians than blacks with a slightly higher incident rate in males in comparison to females (American Cancer Society, 2006 and 2008).

An estimated 62,480 new cases will be diagnosed in 2008 according to the American Cancer Society. The number of new cases among white men and women increased approximately 6% per year from 1973 until the early 1980's. Nonetheless melanoma incident rate has declined since 1981 to less than 3% per year. The increased awareness of melanoma, early detection and the wide use of sunscreen as a precautionary measure against harmful UV rays are some factors that attribute to this decrease. In 2008, the American Cancer Society predicts 8,420 individuals will die from melanoma in the U.S. though death rate from melanoma has been relatively constant since 1990 for Caucasian men and declining since 1988 for Caucasian women. Since 1973, the

mortality rate for melanoma has increased by 50%, in which much of the increase has been in older people, mostly white men. The 5-year survival rate, stage 0, I, II, III, IV melanoma are 97%, 90 to 95%, 45 to 78%, 28 to 70% and 18% respectively (American Cancer Society, 2006 and 2008) .

Melanoma Treatments

Numerous treatment options are available for melanoma. One method is surgical removal by simple excision, re-excision if positive skin biopsy confirms melanoma, amputation, and lymph node dissection if lymph node biopsy indicates malignancy near cancer site (American Cancer Society, 2006). Patients most likely to benefit from lymph node removal are those with soft tissue, lymph node, lung, bowel, adrenal and brain solitary lesions (Atallah and Flaherty 2005). However, early stage diagnosis and treatment reveal the best results.

Chemotherapeutic drugs, serve as alternative approaches for treatment of metastatic malignant melanoma. These drugs interfere with rapidly dividing cells that are beneficial for treating cancer cells that has spread throughout the body. Administered intravenously or orally, numerous adverse side effects are associated with the treatment such as nausea and vomiting, hair loss, loss of appetite, increase infection, peripheral neuropathy, ototoxicity and/or fatigue that are dependent upon the dosage of chemotherapy administered. The side effects usually subside upon completion of treatment (Atallah and Flaherty 2005).

The classes of drugs generally used in melanoma treatment include (i) *alkylating agents* that induce their cytotoxic effects by alkylating DNA within the nucleus that cause cell death include dacarbazine (DTIC), temozolomide, and cisplatin, which destroys all

stages of the cell cycle and prevents DNA synthesis and function; (ii) *plant alkaloid* vinblastine that disrupts cytoskeleton structure hindering tubulin polymerization and mitotic spindle formation and inhibits of mitotic division that results in cell death; and (iii) *antitumor antibiotics* bleomycin that induce single- and double-stranded DNA breaks by interacting with DNA and inhibiting DNA biosynthesis (Katzung, 2007).

Combination of different drug treatments works better than single drug for stage IV melanoma (American Cancer Society, 2006). Many combinations of chemotherapy drugs are used in metastatic malignant melanoma. The resistance of melanoma cells to chemotherapy makes treatment challenging and detrimental. The two most active combinations are the four drug combination of cisplastin (DNA alkylating agent), DTIC (DNA alkylating agent), bischloroethylnitrosourea (BCNU) and tamoxifen (Darmouth regimen) and cisplatin (DNA alkylating agent), vinblastine (mitotic inhibitor) and DTIC (DNA alkylating agent) (Atallah and Flaherty 2005). The Darmouth regimen initially demonstrated a 43% response rate (Lattanzi et al 1995) however there were no differences in overall survival when compared to randomized phase III trial with DTIC only (Chapman et al 1999). Another two chemotherapeutic drugs used in combination are thalidomide (anti-angiogenenic drug) and temozolomide (alkylating agent). Reports indicated that twelve of thirty eight patients responded with one remission and five other patients resected free of disease. The median survival was nine months and treatment was endurable (Hwu et al, 2003).

Recently, a new therapeutic approach involving poly(ADP-ribose) polymerase, PARP (protein associated with DNA repair) inhibitors, have been introduced into clinical trials (Kasper et al, 2007). The application of PARP inhibitors have been used to treat a

variety of diseases such as cancer, cardiac infarct, stroke and diabetes (Woon and Threadgill, 2005; Graziana and Szabo, 2005). PARP can cause development of resistance to chemotherapeutic agents via DNA damage repair and tumor killing effects (Kasper et al, 2007). Blocking this protein makes cancer cells more sensitive to chemo treatment thus, enhancing effects on tumor (Plummer et al, 2005). The use of this inhibitor along with temozolomide are a part of ongoing studies in eighteen patients with newly diagnosed or recurrent unresectable stage III of IV melanoma at the MD Anderson Cancer Center (Kasper et al, 2007).

The concept of immunotherapy is another new developing field of interest for cancer treatment based on the body's immune system to produce immune responses against cancer cells. When foreign materials or pathogen are introduced into the body chemical mediators are secreted to induce an immune response. Cytokines includes lymphokines, interleukins and interferons which are soluble signaling protein and glycoprotein molecules that act as mediators released by activated T-cells to enhance amplification of T-cells and destruction of foreign cells. This principle can be applied in cancer therapy such that the body's own immune system can increase cytotoxic T-cells production against cancer cells. For individuals with advanced melanoma there are several types of immunotherapy used. Cytokine (proteins that turn on the immune system) production in stage III and IV melanomas have been shown to reduce tumors in about 10 to 20% of patients, however adverse effects include fluid build up that results in swelling thus makes a person feel sick (Kasper et al; 2007). There is ongoing combination cytokine therapy research for treating advanced stage melanoma in patients though toxicity poses an obstacle to overcome (Kalaaji, 2007).

Cytotoxic T lymphocyte-associated protein 4 (CTLA) is another target. The presence of self-antigen on tumors cells makes tumor removal ineffective by the immune system hence development of therapeutic strategies involving tumor immunity becomes difficult (Kasper et al; 2007). Studies by Kapadia and Fong (2005) suggest T cells are essential in inducing tolerance and reducing immune responses to tumor antigens by producing CLTA-4. Such findings have lead to the development of antibodies blocking CLTA-4 and clinical trials (Kasper et al 2007). Other reports have indicated the inhibition of T cells by CLTA-4 in immune response (Chambers et al, 2001) whereas, blockage of CLTA-4 increase T cell responses and tumor rejection (Leach et al 1996). The combination of CLTA-4 blockade and tumor vaccination can further improve antitumor activity (Hurwitz et al, 2000). Such findings have lead to the development of antibodies against CTLA-4 and the start of clinical trials.

Heat shock proteins serve numerous functions including mediation of apoptosis, proteasomal destruction, interaction with other proteins and immunological properties (Blachere and Srivastava 1995). The injection of heat shock protein modulators, HspPC-96 in metastatic melanoma patients demonstrated a tolerance to vaccine with minimum toxicological effects in 18% of patients (Pillia et al 2005). However, making a vaccine against tumors such as melanoma is more difficult than making a vaccination to fight a virus.

Other quasi-immune alternative methods in the treatment of malignant melanoma include antiangiogenic drugs, such as the humanized antibody bevacizumab (Avastin) directed against vascular endothelial growth factor (VEGF) and Bcl-2 antisense compound. One such drug is oblimersen which reduces Bcl-2 anti-apoptotic protein in

combination with chemotherapeutic agents, dacarbazine (DNA alkylating agent), paclitaxel (mitotic inhibitor) or interferon (Kasper et al, 2007). The results from ongoing trials are not known yet. Targeting RAF proteins associated with the mitogen-activated protein kinase (MAPK) signal transduction pathway which are involved in regulation of cell proliferation, differentiation and survival serve as another approach. Treatment of melanoma with compound sorafenib (BAY 43-9006, Nexavar) not only inhibits C-RAF and VEGFR, but also targets B-RAF, which has been implicated to be an important target in melanoma to induce apoptosis (Panka et al 2006). Some preclinical trails have implicated treatment with the proteosomal inhibitor bortezomib to be active against melanoma. Proteosome inhibition can enhance alterations in pro-apoptotic activities thereby increasing the sensitivity to immune effector cells (Markovic et al 2005).

Another melanoma cancer treatment includes radiation. Radiation therapy utilizes high energy rays or other ionizing radiation types to reduce or prevent tumor development. Radiation can be applied externally or internally where radioactive substances are contained within needles, seeds, wires, or catheters positioned directly into or close by tumor site. Though this is an uncommon method of treatment for melanoma patients, ionizing radiation can be used for a tumor that has returned in the skin or lymph node that cannot be removed surgically (Corry et al., 1999).

Mechanisms of Programmed Cell Death

Two types of programmed cell death (PCD) include apoptosis (type I) and autophagy (type II). Both involve an intracellular program that leads to cell death. The term "apoptosis" (Greek origin meaning falling or dropping off) is defined as a form of programmed cell death. Many observations since the mid-nineteenth century has shown

the importance cell death plays in physiological processes of multicellular organisms, such as in embryological development and metamorphosis (Glucksmann, 1951; Lockshin, 2001). The term "programmed cell death" was initially introduced in 1964 (Locksin, 1964), however the morphological processes that resulted from cellular destruction were first defined in publication by Kerr et al., in 1972. Apoptosis is characterized by several morphological changes including cell shrinkage and condensation, DNA fragmentation, cytoskeletal collapse, disassembly of the nuclear envelope, externalization of phosphatidylserine, and biochemical changes such as caspase activation, changes in pro- and anti-apoptotic protein levels and cytochrome c release from the mitochondria. Although the presence of many of these hallmark indicators can suggest an apoptotic type of cell death, there are different pathways defined that may or may not include each apoptotic event.

The induction of apoptosis can occur via three well-characterized pathways, intrinsic, extrinsic or perforin/granzyme. The distinction among these three relies in part on the difference in activated enzymes and proteins that participate in the degradation of the cell. Caspases are intracellular proteolytic enzymes that exist as inactive zymogens. These enzymes are an essential component of apoptosis responsible for the breakdown of cellular proteins. Various triggers can lead to their activation, which usually occurs through proteolytic processing of the zymogen at conserved aspartic acid residues. The caspases initially involved in the apoptotic process are known as "initiators" (-8, -9, and 10) that cleave and activate executioner caspases (-3, -6 and -7) that induce cell death (Elmore, 2007).

The intrinsic pathway becomes activated in response to intracellular stresses such as DNA damage, serum deprivation, or stress to endoplasmic reticulum (Adams, 2003; Aoki, 1996). These stimuli elicit alterations in the mitochondrial membrane that cause mitochondrial permeability transition pore (MPT) opening and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) that leads to the release of two main groups of pro-apoptotic mitochondrial proteins. The first group include cytochrome c, second mitochondria-derived activator of caspase (Smac), direct IAP-binding protein (DIABLO), and serine protease HtrA2/Omi (Elmore, 2007). The loss of $\Delta\Psi_m$ and pore openings in mitochondrial membrane results in the release of mitochondrial protein cytochrome c, a soluble protein localized in intermembrane space loosely attached to the inner mitochondrial membrane. In the cytosol, the interaction of cytochrome c, adaptor protein, apoptosis-proteases-activating-factor-1 (APAF-1) and procaspase -9 in the presence of dATP forms an apoptosome. This proteolytically activates caspases -9 then -3 to execute apoptotic death by digesting essential cellular proteins such as activation of DNA degrading enzyme DNAse via cleavage resulting in DNA fragmentation (Albert, 2002; Jacobson and McCarthy, 2002). The Smac/DIABLO and HtrA2/Omi mitochondrial proteins interact with apoptosis inhibitory proteins (IAP) to neutralize anti-apoptotic activity and induce cell death (Hengartner, 2000; van Loo et al., 2002; Schimmer, 2004).

The second group of pro-apoptotic mitochondrial proteins consists of apoptosis inducing factor (AIF), endonuclease G and caspase activated DNase (CAD) that are released in the late apoptotic phase once the cell death is evitable. These proteins translocate to the nucleus and initiate oligonucleosomal DNA fragmentation and

enhanced chromatin condensation. Both AIF and endonuclease G function independently of caspase activation whereas CAD requires initial cleavage by caspase-3 which results in oligonucleosomal DNA fragmentation (Elmore, 2007).

The Bcl-2 family of proteins plays a critical role in the control and regulation of the mitochondria involvement in the stress-induced apoptosis process (Elmore, 2007). This family of pro- and anti- apoptotic proteins are categorized into three subfamilies based on sequence and protein function: (i) Bcl-2, anti-apoptotic proteins that exert cell death activity and shared sequence Bcl-2 homology (BH) within four regions. Examples include Bcl-2, Bcl-x_L, and Bcl-w; (ii) Bax, pro-apoptotic proteins that have homology only within BH 1 to 3 and not 4 region and includes Bax, Bak, and Bok; and (iii) BH3-only, which are pro-apoptotic proteins that share sequence homology only within BH3 such as Bik, Bid, Bad, Bim, p53 upregulated modulator of apoptosis (PUMA) and Noxa (Bouillet and Strasser, 2002; Jacobson and McCarthy, 2002). The sensitivity of cells to apoptotic stimuli can depend on the balance between pro- and anti- apoptotic Bcl-2 proteins. The increase in pro-apoptotic protein levels such as Bax and Bid disrupts the normal function of the anti-apoptotic Bcl-2 proteins that leads to the loss of $\Delta\Psi_m$ and pore formation in the mitochondria. It has been proposed that Bax forms a transmembrane pore in the outer membrane of mitochondria, resulting in the loss of membrane potential and cytochrome c release (Albert, 2002; Jacobson and McCarthy, 2002). Under non-apoptotic conditions, the overexpression of anti-apoptotic proteins, Bcl-2 and Bcl-x_L regulate cell death induction by inhibiting cytochrome c release from the mitochondria and pro-caspase-9 activation (Elmore, 2007).

While other Bcl-2 family members play roles in both the intrinsic and extrinsic pathways, Bcl-2 family members of the pro-apoptotic proteins, PUMA and Noxa are primarily associated with the intrinsic pathway. The DNA damage that initiates apoptosis usually requires tumor suppressor gene *p53*, which activates gene transcription that encodes a protein to promote cytochrome c release. Both activation of PUMA and Noxa are triggered by *p53* as a result of geno-toxic damage or oncogene activation (Elmore, 2007). PUMA induces a *p53* mitochondria dependent apoptotic pathway together with enhanced Bax expression that causes cytochrome c release and subsequent caspase -9 and -3 activation, respectively (Nakano and Vousden, 2001; Liu et al, 2003). Noxa on the other hand, localize to mitochondria also mediates *p53* apoptotic induction by caspase -9 activation (Oda et al., 2000).

The extrinsic signaling pathway operates through death receptors (Fas, tumor necrosis factor (TNF), TNF- apoptosis inducing ligand (TRAIL) of the tumor necrosis factor (TNF) superfamily and their corresponding signaling molecules, ligands (TNF, FasL, and TRAIL). TNF-R1 family of receptors can interact with one or more ligands that bind with type II transmembrane receptors either soluble or membrane bound. Ligand trimerization occurs initially to facilitate ligand interaction and activation of receptor that induce cell death, proliferation or differentiation (Jacobson and McCarthy, 2002).

The extrinsic pathway consists of a mitochondrial-independent (type I) and mitochondrial-dependent (type II) apoptotic induction. The extrinsic type I pathway is initiated by activation of the death receptors and their death ligands. The death domain of the death receptor recruits death adaptor proteins (Fas-associated protein with death

domain, FADD or TNF receptor-death domain, TRADD) along with pro-caspase-8 enzyme and forms the death inducing signaling complex (DISC). The cleaved active caspase-8 fragment then activates caspase -3. This apoptotic pathway can be inhibited by c-FLIP (cellular FLICE-like inhibitory protein) by blocking activation of caspase -8 thereby preventing caspase -8 recruitment to DISC (Thome et al., 1997; Irmler et al, 1997). The extrinsic type II pathway utilizes death receptors, however, caspase -8 cleaves pro-apoptotic protein BID which acts on the mitochondria to release cytochrome c thus activating caspase -9 then -3. Thus the intrinsic and extrinsic mechanisms are not independent and are connected as indicated by the role of t-Bid.

Ultimately, both the intrinsic and extrinsic apoptotic pathways lead to the activation of executioner caspases -3, -6 and -7 that are essential for executing the final apoptotic phase. At this stage cell death is inevitable demonstrated by morphological changes distinct for the classification of apoptosis. Caspase -3 plays a major role in mediating proteolysis of numerous substrates involved in triggering endonucleases and cytoskeleton breakdown. Caspase -3 is correlated with the activation of caspase activated DNase (CAD) endonucleases responsible for DNA fragmentation by cleaving inhibitor segment of the inhibitor caspase activated DNase (ICAD). The disruption of nuclear and plasma membrane associated cytoskeleton proteins, fodrin, vimentin, lamin A and gelsolin essential for actin filament assembly along with cleavage of substrates, poly (ADP-ribose) polymerase (PARP) involved in DNA repair mechanism, signal transducers and activators of transcription protein-1 (STAT1) and X-linked inhibitor of apoptosis protein (X-IAP) are also targeted by executioner caspase -3 to complete apoptosis (Slee et al., 2001; Martin et al., 1995; Elmore, 2007).

The perforin/granzyme pathway is another apoptotic pathway utilized mainly by cytotoxic T lymphocytes (CTLs) by way of the extrinsic pathway and FasL/FasR interaction. CLTs elicit cytotoxic effects to destroy tumor and virus-infected cells through perforin (transmembrane-pore forming molecule) secretion in conjunction with cytoplasmic granules release through the pore and into target cell. These cytoplasmic granules consist of two important serine proteases granzyme A and granzyme B. Granzyme B operates by cleavage of proteins at aspartate residues that lead to procaspase-10 activation that can cleave ICAD which results in DNA fragmentation or through a mitochondrial apoptotic pathway via Bid cleavage that induce cytochrome c release followed by caspase activation or directly to activate caspase -3 (Barry and Bleackley, 2002; Russell and Ley 2002).

Granzyme A is an essential part of CLT induced cell death mechanisms that activate caspase independent pathways. This protease enters the cell and trigger DNA fragmentation via DNAse NM23-H1, a tumor suppressor gene product associated with apoptosis induction in cancer tumor cells. Granzyme A interacts with nucleosome assembly SET complex of proteins which includes SET, Ape1, pp32 and HMG2 that inhibit NM23-H1. The activation of NH23-H1 occurs via granzyme A protease cleavage of SET complex and NM23-H1 releasing NM23-H1 that leads to DNA degradation and apoptosis. The SET complex has additional functions that involve chromatin and DNA structure protection however inactivation of complex blocks DNA maintenance and chromatin structure that attributes to apoptosis (Elmore 2007).

Autophagy, is another form of programmed cell death (PCD) by which cells recycle cytoplasm and dispose of defective organelles. This type of PCD is

characterized by autophagic vacuole (AV) formation that result in intracellular organelle (Golgi apparatus, polyribosomes and endoplasmic reticulum) destruction preceding nuclear collapse (Bursch et al., 1996). The maintenance of cytoskeletal structure is vital for this process to occur. Death-associated protein (DAP)-kinases and related protein kinase (DRP-1) are major regulators in autophagic PCD through enhancement of the mitochondrial membrane permeabilization. These kinases can also be involved apoptotic PCD responsible for membrane blebbing (Bursch, 2004).

Mechanism of Non-Programmed Cell Death

Non-programmed cell death mechanisms that differs significantly from PCD type I and type II include necrosis. Necrosis is a form of cell death primarily linked to inflammatory responses. Cells undergoing necrosis release their intracellular contents into the extracellular environment due to the loss of plasma membrane integrity which initiates an inflammatory response. Typical morphological characteristics are swelling of cells, loss of membrane integrity and cell lysis (Proskuryakov et al., 2003). Necrotic cell death can be induced by various viruses, bacteria and protozoa which can activate bacterial toxins, and component of the immune defense such as complement, activated natural killers and peritoneal macrophages (Warny et al., 2000; Dong et al., 1997; Shimizu et al., 2000; Blom et al., 1999; Arantes et al., 2000).

Importance of Apoptosis in Cancer Treatment

The induction of apoptosis is involved in many important biological processes. One such process includes reproductive organ development that differs for males and females. During embryonic development for example, the Müllerian duct forms the uterus and oviduct in females however these organs are not developed in males and are

removed by apoptosis (Meier et al., 2000). Another example is lymphocyte development, maintenance of homeostasis and disease. Increase apoptotic induction in lymphocytes can lead to immunodeficiency whereas inhibition can cause autoimmunity or lymphoma (Rathmell and Thompson, 2002). Also elimination of damaged and harmful cells, such as irreparably DNA-damaged cells, autoreactive cells, and infected cells (Gewies, 2003).

On the other hand, dysfunctions in the apoptotic pathway have been associated with numerous pathological conditions. Such mutations or defects can result in cancer, autoimmune diseases and the spreading of viral infections whereas excessive apoptosis can cause neurodegenerative disorders, AIDS, and ischemic diseases (Reed, 2002; Fadeel, 1999). Mutations in apoptotic pathway has also been implicated in plant biology by activation cysteine proteases (Solomon, 1999) and yeast (Frohlich, 2000; Skulachev, 2002).

Mutations in the apoptotic pathway have been associated with tumorigenesis and progression, metastasis, and development of resistance to chemotherapeutic drugs (Johnstone et al., 2002). The development of mutations in adaptor protein APAF-1, RAF family of protein BRAF, tumor suppressor gene *PTEN* and cyclin-dependent kinase family CDK4 have been associated with melanoma (Haluska et al., 2006). The downregulation of pro-apoptotic protein PUMA and upregulation of apoptotic inhibitors *survivin* and FLIP can attribute to this phenomenon (Irmler et al., 1997; Grossman et al., 1999; Soengas et al., 2001; Karst et al., 2005). The proliferation rate of tumor development is not merely due to oncogene activation but impairment of apoptosis checkpoint (Hanahan and Weinberg, 2000; Wang, 1999). Therefore providing

appropriate treatment conditions to inhibit or activate pro- and anti-apoptotic proteins can provide effective treatment in cancer treatment.

Agents That Induce Apoptosis in B16F10 Melanoma Cells

Many compounds have demonstrated different mechanisms in the induction of apoptosis in B16F10 melanoma cells. Capsaicin which is a pungent ingredient found in hot chilli peppers that have been noted for its anticarcinogenic and antimutagenic activities. This compound was reported to inhibit growth and induce apoptosis marked by nuclear condensation, internucleosomal DNA fragmentation, enhanced sub G1 fraction, cytochrome c release, caspase -3 activation and cleavage of poly (ADP-ribose) polymerase. Though Bcl-2 level were slightly down regulated, no changes in Bax levels were detected (Jun et al., 2007)

Aspirin (salicylate and acetyl salicyclic acid) was revealed to reduce B16F10 melanoma cells proliferation rate dependent on c-Jun N-terminal kinase (JNK) (Ordan et al 2003). The extracellular matrix (ECM) is known to be essential in the survival and metastasis of cancer cells. Treatment of B16F10 cells with methylselenol disrupted integrin expression and ECM adhesion through a caspase induced cell death mechanism (Kim et al., 2007). Also vitamin C was shown to stimulate a caspase -8 independent apoptosis by phosphatidylserine externalization, enhanced levels of reactive oxygen species (ROS), decrease in mitochondrial membrane potential and cytochrome c release (Kang et al., 2003).

Blood supply is essential in tumor development. The use of angiogenic inhibitors can be beneficial in cancer therapy as the formation of new blood vessels is required for development. The angiogenesis inhibitor, TNP-470, induced cell death two hours post

treatment without typical apoptotic morphological changes identified, however reactive oxygen species (ROS) were generated (Okroj et al., 2005). Another study investigated further the effects of TNP-40 in B16F10 melanoma cells. The results demonstrated uncharacteristic cell death, with no phosphatidylserine externalization but membrane permeability indicated by propidium iodide uptake. No mitochondrial transmembrane potential changes detected and no effect of the panspecific caspase inhibitor (Okroj et al., 2006).

Electric Field Application in Cancer Treatment

The application of electric fields to mammalian cells is currently being used in cancer therapy. Convectional electroporation has been around for decades and electroporation is an alternative approach considered in cancer treatment. This method delivers long electrical pulses ranging from milliseconds to microseconds that enhances ion conductance across the plasma membrane through creating large pores (Weaver, 1995). The application of electric pulses, permeabilize cells that facilitate the entry of DNA, RNA, drugs and proteins into the cells. The basis of this method has lead to the incorporation with other therapeutic techniques to enhance the efficacy of cancer treatment and chemotherapeutic drugs. Electrochemotherapy (ECT) is one such method that incorporates the application of both electric fields and chemotherapeutic drugs by introducing drugs into the cell and enhancing therapeutic effects. The application of ECT was demonstrated in combination with chemotherapeutic drug bleomycin that resulted in tumor reduction in mice (Mir et al., 1991a). The results of this study lead to ECT treatment in combination with bleomycin clinical trials that was effective in treatment of head and neck squamous carcinomas in patients (Mir et al., 1991b).

Belehradek et al. 1993 demonstrated effective treatment initially in melanomas ECT with bleomycin on cutaneous metastases of head and neck carcinoma patients. Early clinical trials by Heller et al. 1996 used ECT with bleomycin in treatment of malignant melanoma, two with basal cell carcinoma, and one with metastatic adenocarcinoma. The most effective response was demonstrated in adenocarcinoma with complete responses in both treated nodules after ECT. The application with bleomycin has been successful in the treatment of cutaneous, subcutaneous and perineal malignant tumors. The introduction of chemotherapeutic drugs into tumor cells, such as bleomycin, has demonstrated beneficial results in the treatment of melanoma tumors (Kubota et al, 2005; Gothelf et al, 2003).

ECT technique has been implicated to enhance the efficacy of immunotherapeutic drugs in cancer treatment. The amplification of cytokines, more specifically interleukins in combination with electroporation can potentially serve an alternative approach for melanoma treatment. Study by Dev and Hofmann, 1994 initially paved the way for this approach illustrating the amplification of interleukin-2 (IL-2) in effective treatment of squamous cell carcinomas of the head and neck in animals and patients. More recent report in B16 mouse melanoma demonstrated interleukin-12 (IL-12) plasmid delivery along with electroporation reduce tumor revealing minimum adverse side effects associated with it (Heller et al., 2006).

Irreversible electroporation is another procedure involving electrical fields that can be potentially used on tumor treatments. Such application involves permanent permeabilization of the membrane that alters cell homeostasis that leads to cell death (Rubinsky, 2007). The potential application of irreversible electroporation treatment has

been highlighted in liver cancer and prostate cancer though scarring from ablated area results due to heating (Lee et al., 2007; Onik et al., 2007). Radiofrequency thermal ablation (RFA) is somewhat new treatment approach that produces alternating currents that generates ionic agitation that leads to frictional heating to destroy tumors. Numerous types of carcinomas (renal, hepatic, prostate, bone and others) have been treated by RFA however optimal results have been demonstrated in breast cancer patients (Fornage et al., 2004; Vlastos and Verkooijen HM, 2007). Though all previously described electrical pulse applications demonstrate an effective means of carcinoma treatment, they all generate heat that cause tissue damage and scarring to ablated area. For both irreversible electroporation and radiofrequency ablation the mechanism for tumor death is necrosis.

Advantage of New Nanosecond Pulsed Electric Fields (nsPEF) Treatment

The application of nsPEF differs from classical electroporation which has longer pulse durations (milli- to micro-second) with charging time of the plasma membrane as oppose to shorter durations of nanosecond pulses that generates pulses with high-intensity electric fields (Beebe et al, 2003a). Unlike electroporation, these nanosecond pulses deliver high electric field intensity (kV/cm) and high power (megawatts) but low energy density (mJ/cc) (Hall et al, 2005) which generates little heat. Also in contrast to electroporation, which produces large pores in the plasma membrane that permit entry of large molecules, drugs or genes, nsPEFs produce large number of nanopores that limit entry to ions but not drugs or genes. The field can penetrate into the cell permeabilizing intracellular organelles (Schoenbach et al, 2001) and mobilizing calcium (White et al, 2004; Beebe et al, 2003b; Vernier et al, 2003; Vernier et al, 2006). These ultra-short pulses can act as non-ligand agonists that induce cell signal transduction mechanisms that

induce apoptosis by affecting intracellular and plasma membrane structures and functions. Reports in Jurkats have illustrated that nanosecond pulses induce direct electric field and biological effects that initiate apoptosis and caspase activation as indicated in Jurkats and HL-60 cells (Beebe et al, 2003a). Electric field target intracellular organelles that subsequently lead to apoptosis induction *in vitro* (Beebe et al, 2003b). DNA fragmentation (Stacey et al, 2003; Hall et al., 2007) and reduced effects on the plasma membrane with enhanced functions on intracellular structures has also been demonstrated with nsPEF application (Schoenbach et al, 2001; Beebe et al 2003a). However, it is interesting that nsPEFs induced apoptosis in p53 null and wildtype HCT 116 cells, suggesting that if DNA is damaged, p53 does not play a role.

Other advantages of nsPEFs have been demonstrated in tumor reduction (Beebe et al 2002; Nuccitelli et al., 2006). The study in B16F10 mouse melanoma tumor suggest that nanosecond pulses induce apoptosis and tumor regression generating little to no thermal buildup and require no administration of chemotherapeutic agents, which reduces adverse side effects induce by chemotherapy. Furthermore the blood supply to the tumor is reduced and there is reduced/no scarring potential as opposed to the surgical skin lesion removal that leaves skin scars. NsPEFs are minimally invasive and are more likely to affect the tumor without disrupting the dermis (Nuccitelli et al, 2006). Though results of this study are promising for nanosecond pulses as an alternative cancer therapeutic approach, however, it will be important to determine the cell death mechanism(s) associated with this observation, which require additional studies and are addressed in this dissertation. Although other studies have implied activation of multiple apoptotic pathways induced by nsPEFs in different cell types (Beebe et al., 2003a; Hall et al.,

2007), this thesis will demonstrate the induction of multiple apoptotic pathways induced the nanosecond pulses in the a single cell type, B16F10.

Previous/Current Publications

Previous work has indicated reduction in fibrosarcoma tumors in mice when electric fields are applied (Beebe et al, 2002). Current study from the Old Dominion University Frank Reidy Center for Bioelectrics has shown that the administration of nanosecond pulsed electric fields reduce melanoma tumors by 90% within two weeks of electric field application via restriction of blood supply and reduction in cell nuclei (Nuccitelli et al, 2006). Even though a 2.6-fold increase of caspase activity was mentioned for B16F10 melanoma tumors, no statistical significant differences were detected (Nuccitelli et al, 2006). The stated reason for this finding was that apoptosis is an energy-requiring process and restriction of blood does not allow the apoptosis to go to completion (Nuccitelli et al, 2006). However, more recent unpublished studies have demonstrated that nsPEFs induce increases inn the numbers of apoptotic cells in B16F10 melanoma tumors *in vivo* as determined by fluorescent antibodies to caspases-3, -6, and -7.

SPECIFIC AIMS

Specific Aim 1: To determine whether nsPEF stimulation induces apoptosis in B16F10 melanoma cells *in vitro*.

This aim determined whether nsPEFs induce apoptosis in B16F10 melanoma cells *in vitro*. The first hypothesis was that nsPEFs affect viability/survivability in a time and electric field dependent manner. The second hypothesis was that nsPEFs effects membrane integrity and phosphatidylserine externalization in an electric field dependent time course. The third hypothesis was nsPEFs induce caspase activation in an electric

field dependent manner independent of calcium mobilization. The fourth hypothesis was nsPEFs induce apoptotic morphological changes such as F-actin cytoskeletal disruption and membrane blebbing.

Specific Aim 2: To establish whether nsPEFs stimulate a mitochondrial-independent or -dependent apoptotic pathway

This aim established whether nsPEFs stimulate a mitochondrial-independent or -dependent apoptotic pathway. The fifth hypothesis was that nsPEFs induce caspase-dependent apoptosis in the absence of cytochrome c release, mimicking the type I extrinsic apoptosis pathway. The sixth hypothesis was nsPEFs induce activation of initiator caspase -8 and executioner caspases -3, -6 and -7.

SIGNIFICANCE

The aim of the project is not only to understand how nsPEFs induce cell death and the pathway mechanisms by which cells undergo apoptosis *in vitro* but also to provide an understanding for the application of pulse electric fields. Though it is not fully understood how these tumor cells undergo cell death, but some melanoma cells are known to require calcium and contain mutations in their apoptotic pathway (Niedojadlo et al, 2005). Investigation into how nsPEFs induce cell death and the multiple apoptotic pathway(s) involved in this process is essential in understanding nsPEFs role in the initiation of cell death in these melanoma cells. This can potentially provide an alternative non-ligand, non-chemotherapeutic treatment of melanoma tumors (other malignant tumors) or an alternative combination treatment that consists of nsPEFs and low-dose chemotherapeutic treatment reducing side effects that result from high dose treatments.

CHAPTER II

NANOSECOND PULSED ELECTRIC FIELDS INDUCE APOPTOSIS IN B16F10 MELANOMA CELLS *IN VITRO*

INTRODUCTION

Apoptosis is a form of programmed cell death that is mediated by caspases, a family of cysteine proteases. Caspases are intracellular proteases that exist as catalytically inactivate zymogens that are required to undergo proteolytic cleavage and activation during apoptosis. These enzymes are an essential component responsible for apoptosis induction and the morphological changes that lead to cell death. Initially, apoptosis induction requires the initiation of cellular events to activate apoptotic mechanism such as intracellular stresses, receptor-mediated signals, cytotoxic T cell attack or some other inducer. Once cell death inevitably commits to cell death by activation of executioner caspases or release of mitochondrial proteins from the intermembrane space the cell apoptotic degradation process takes place. This degradation event is related to the final removal of the cell, including plasma membrane blebbing, cell shrinkage, DNA fragmentation and phosphatidylserine externalization (Wilson, 1998; Elmore, 2007).

Nanosecond pulsed electric fields (nsPEFs) are ultrashort pulses with high electric field intensity (kV/cm) and high power (megawatts) but low energy density (mJ/cc) (Hall et al, 2005). Previous *in vitro* studies have implicated these ultra-short pulses induce direct and biological effects that initiate apoptosis at the plasma membrane and intracellular structures. Studies in Jurkat T lymphoblasts and HL-60 cells illustrated

nsPEF stimulate effects on the plasma membrane through externalization of phosphatidylserine and enhanced membrane permeabilization (Beebe et al, 2003a; Vernier et al., 2004). The identification of intracellular apoptotic proteins, caspase FITC-VAD-FMK and pro-apoptotic protein BAX have been identified with administration of nsPEFs (Beebe et al, 2003a; Vernier et al., 2003; Hall et al., 2007). Such treatment has been shown to cause cell shrinkage and DNA fragmentation correlated with apoptosis (Stacey et al., 2003; Hall et al., 2007). The effects on intracellular organelles via intracellular calcium release have been reported to induce caspase activation that leads to cell death (Mattson, 2003). The mobilization of calcium initiated by nsPEFs has been shown; however, caspase activation can be dependent and independent of calcium (Beebe et al., 2003).

Previous reports suggest that nsPEFs induce apoptosis in Jurkat T lymphocytes, HL-60, HCT 116p(+/+) and HCT116p53(-/-) cells. Here the objective was not solely to evaluate apoptosis using standard apoptotic markers to examine plasma membrane and intracellular modifications, but also to observe structural morphological changes in the plasma membrane and cytoskeleton structure in B16F10 melanoma cells *in vitro* by way of nsPEFs application. This study revealed induction of apoptosis in a pulse number and electric field dependent manner, independent of calcium mobilization and phosphatidylserine externalization. Administration of treatment also results in disruption of the actin cytoskeleton structure and plasma membrane blebbing as a result of apoptosis.

EXPERIMENTAL PROCEDURE

Cell Culture

Mouse melanoma cells (B16F10) used in study was obtained from ATCC (Manassas, VA). B16 cells were grown in Dulbecco's Modified Eagle's Medium with L-glutamine (ATCC; VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals; Nocross, GA), 1% L-glutamine and 1% penicillin streptomycin (Mediatech Cellgro; VA) and placed in incubator at 37°C with 5% CO_2.

Cell preparation

B16F10 cells for treatment were detached from cultured flask when cells are about 80-90% confluent. This involves removal of media from flask, a rinse with Hank's Balanced Salt Solution 1X (Mediatech Cellgro;VA), trypsin EDTA 1X 0.25%trypsin/2.21mM EDTA in HBSS addition (Mediatech Cellgro;VA) and DMEM culture medium addition. B16F10 cell preparation prior to pulse involves centrifugation at 1000rpm and addition of Dulbecco's Phosphate Buffered Saline (DPBS) solution which contains 0.5mM $CaCl_2.2H_2O$, 4.2mM KCl, KH_2PO_4, 0.5mM $MgCl_2.6H_2O$, 153mM NaCl, 9.5mM $Na_2HPO_4.7H_2O$ and 2.5mM glucose, pH 7.4. Cell count performed using a hemacytometer, centrifugation at 1000 rpm and DPBS solution addition based on $7.7x10^6$cells/mL.

Administration of nsPEF

Trypsinized B16 cells $7.7x10^6$cells/mL are exposed to nsPEF conditions at a concentration of $1x10^6$cells/130μL placed in 0.1cm gene pulser cuvettes (Bio-Rad Laboratories; Hercules, CA). Rectangular high voltage pulses of 300 ns duration and amplitudes of 1.2-6.0 kV are applied by transmission line type pulse generator to the

plane parallel electrodes in the cuvette. Representative pulse shapes are shown in Figure 1. The 10-Ohm impedance of the pulse generator is matched to the resistance of the exposed sample. With increasing voltage, the rise time of the pulses increases slightly from 15.3 to 19.1 ns. The applied voltages correspond to homogeneous electric fields of 12-60 kV/cm in the cuvette.

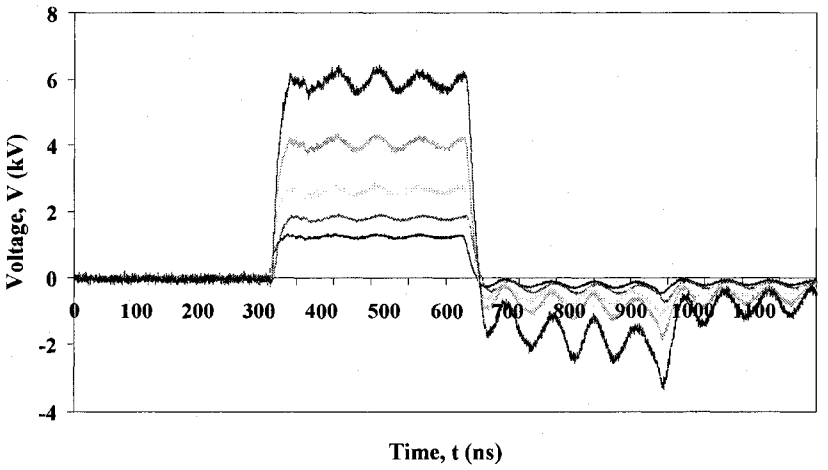

FIGURE 1 Representative graph of voltages applied across B16F10 melanoma cells ranging 12-60kVcm (recorded with Tektronix oscilloscope TDDS32 and Tektronix high voltage probe PA6013).

Trypan Blue Exclusion at 24hrs

B16F10 cells were prepared as previously mentioned and were counted using a hemacytometer prior pulse administration. After 300ns pulse application, cells were transferred from cuvettes into a culture flask with DMEM, 1% L-glutamine and 1% penicillin streptomycin and placed in incubator at 37°C with 5% CO_2 for 24hrs. Cells were detached from the cultured flask as previously mentioned, centrifuged and

resuspended in DPBS. Cell counts were performed using a hemacytometer diluted with trypan blue 24hrs post pulse.

Assessment of Caspase Activation

B16F10 melanoma cells were administered one, three and ten 300ns pulses at 12-60kV/cm in 0.1cm electrode gene pulser cuvettes. Caspase activity was determined with a FITC-VAD-FMK (Promega; Madison, MI) fluorescent cell permeable irreversible inhibitor that binds to the active site of caspases for twenty minutes prior to completion of time course. The cells were washed and re-suspended in DPBS. Fifteen thousand cells were acquired by Becton-Dickinson flow cytometer. Data was analyzed by Cell Quest Pro software.

Evaluation of Yo-Pro-1

B16 melanoma cells were prepared (as mentioned previously) and exposed to ten 300ns pulses in the presence of 0.2μM Yo-Pro-1 (Invitrogen; Oregon) a carbocyanine nucleic acid stains that identifies apoptotic cells while remaining impermeant to propidium iodide. Cells were incubated for fifteen minutes, washed, centrifuged and re-suspended in DPBS solution. The fluorescence intensity measured by a Becton-Dickinson FACSCalibur flow cytometer acquiring 15,000 cells and data analyzed by Cell Quest Pro software.

Evaluation of Membrane Permeability

B16 melanoma cells were prepared as mentioned previously and exposed to nsPEF conditions in the presence and absence of 1:5 propidium iodide/RNase A staining buffer (Invitrogen; Oregon). The fluorescence excitation and emission maximum is 535nm and 617nm, respectively. Fifteen thousand cells were acquired and red

fluorescence analyzed by a Becton-Dickinson FACSCalibur flow cytometer. Data analyzed by Cell Quest Pro software.

Annexin V Binding Examination

B16 melanoma cells were prepared as mentioned previously. The detached cells were exposed to nsPEFs, administered one, three and ten 300ns pulses, centrifuged, re-suspended in Annexin-V binding buffer followed by Annexin-V-FITC incubation (Alexis Biochemical; San Diego, CA). Fifteen thousand cells were analyzed by Becton-Dickinson FACSCalibur flow cytometer one hour post pulse. Data analyzed by Cell Quest Pro software.

Caspase & Calcium Assessment

Calcium levels were measured using 5μM Fluo-3AM (Invitrogen; Oregon), a fluorescent calcium indicator. B16F10 melanoma cells were pre-incubated with Fluo-3AM in the absence and presence of calcium chelators at 20μM BAPTA-AM and 1mM EGTA for thirty minutes, washed and re-suspended in DPBS with or without calcium in 1mM EGTA. B16F10 melanoma cells were administered ten 300ns pulses at 12-60kV/cm in 0.1cm electrode gene pulser cuvettes, washed in re-suspended in DPBS with or without calcium in 1mM EGTA (5μM concentration of ionomycin was used as positive control). Fifteen thousand cells were analyzed by a Becton-Dickinson flow cytometer. Data were analyzed by Cell Quest Pro software.

Caspase activity was determined using a FITC-VAD-FMK indicator (Promega; Madison, WI), a cell permeable irreversible inhibitor that binds to the active site of caspases. B16F10 melanoma cells were first pre-incubated in the absence and presence of calcium chelators at 20μM BAPTA-AM and 1mM EGTA for thirty minutes, washed

and re-suspended in DPBS with or without calcium in 1mM EGTA. Cells were administered ten 300ns pulses at 12-60kV/cm in 0.1cm electrode gene pulser cuvettes, washed in re-suspended in DPBS with or without calcium in 1mM EGTA followed by incubation with FITC-VAD-FMK indicator for twenty minutes. Fifteen thousand cells were analyzed by a Becton-Dickinson flow cytometer. Data were analyzed by Cell Quest Pro software (5μM concentration of ionomycin was used as positive control).

Evaluation of F-actin Cytoskeletal Structure

Cells were prepared and exposed to nsPEF condition as previously mentioned. Cells were fixed according to manufacturer's instructions using fixative from Carboxyfluorescien FLICA Kit (Immunochemistry Technology LLC; Minnesota) for fifteen minutes, dried on slides overnight in incubator at 37°C with 5% CO_2. Cells were permeabilized with 0.2% triton, blocked in 1% Bovine Serum Albumin followed by incubation with 6.6μM rhodamine phalloidin (Invitrogen; Oregon) which stains the F-actin cytoskeleton structure. Slides were cover slipped and mounted with 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and cytoskeletal structure and nucleus viewed by Olympus BX51 microscope and images captured by DP70 camera.

Detection of Membrane Blebbing

Slides were prepared as described above for the evaluation of F-actin cytoskeleton structure except brightfield images of cells viewed by Olympus BX51 microscope and images captured by DP70 camera.

Statistical Analysis

Statistical analysis was performed using SigmaPlot software. Non-nominal data were compared using two-way ANOVA followed by Bonferroni t-test. This test was

used to analyze significant differences in caspase activity between groups pulsed in the presence and absence of calcium chelators (Figure 8). A p value of <0.05 was considered significant.

RESULTS

NsPEF-induced cell death is correlated with caspase activation in a pulse number- and electric-field manner in B16 melanoma cells.

In order to determine if nsPEFs induced apoptosis in B16F10 melanoma cells in culture, cells survivability 24 hours post pulse via trypan blue exclusion and caspase activity measured by flow cytometry was examined. For one or three pulses at electric fields between 12-60 kV/cm there was no significant decrease in cell survival 24 hours post pulse (Figure 2A) and no increase in caspase activity (Figure 2B). However, for one and three pulses at 40 or 60kV/cm cell numbers did not increase either because the cell cycle progression was delayed or the number of cells that divided equaled the number of cells that died. When cells were exposed to ten 300ns pulses, decreases in cell survival and increases in caspase activation were coincident in an electric field-dependent manner, with significant changes in both parameters evident at 40 and 60kV/cm. Caspase activation was observed as early as 30 minutes post pulse (data not shown).

Increases in the nanosecond pulse electric field recruits greater number of cells to induce caspase activity.

Unknown whether nsPEF effects on caspase activity were due to increases in activity in sub-population of cells or an increase in the numbers cells expressing activity as the electric field increased further evaluations were necessary. To determine this B16F10 melanoma cells were exposed to ten 300ns pulses with increasing electric fields

and the entire population analyzed for the percentage of cells that showed active caspase by flow cytometry. The individual panels in Figure 2 representative of a typical experiment displayed in histogram illustrate results from a typical experiment showing active caspase-negative and caspase-positive cells on the left and the right side of the line respectively. As the electric field is increased greater percentages of cells are recruited into the caspase-positive population.

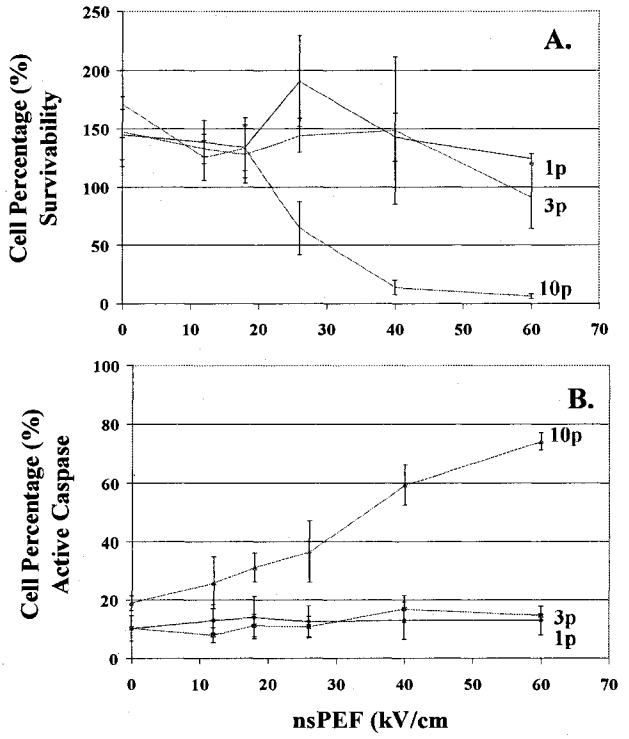

FIGURE 2 NsPEF-induce cell death is correlated with caspase activation in a pulse number- and electric-field dependent manner in B16F10 melanoma cells. Panel A: B16 melanoma cells were exposed to one (n=3), three (n=3) and ten (n=5) 300ns pulses ranging from 12-60kV/cm and survivability was assessed by trypan blue exclusion 24 hours post pulse. Panel B: Active caspase fluorescence intensity is indicated as cell percentage measured using a FITC-VAD-FMK *In Situ* Marker 2 hours post pulse (n=3). Cells were analyzed by flow cytometry 2 hours post-pulse.

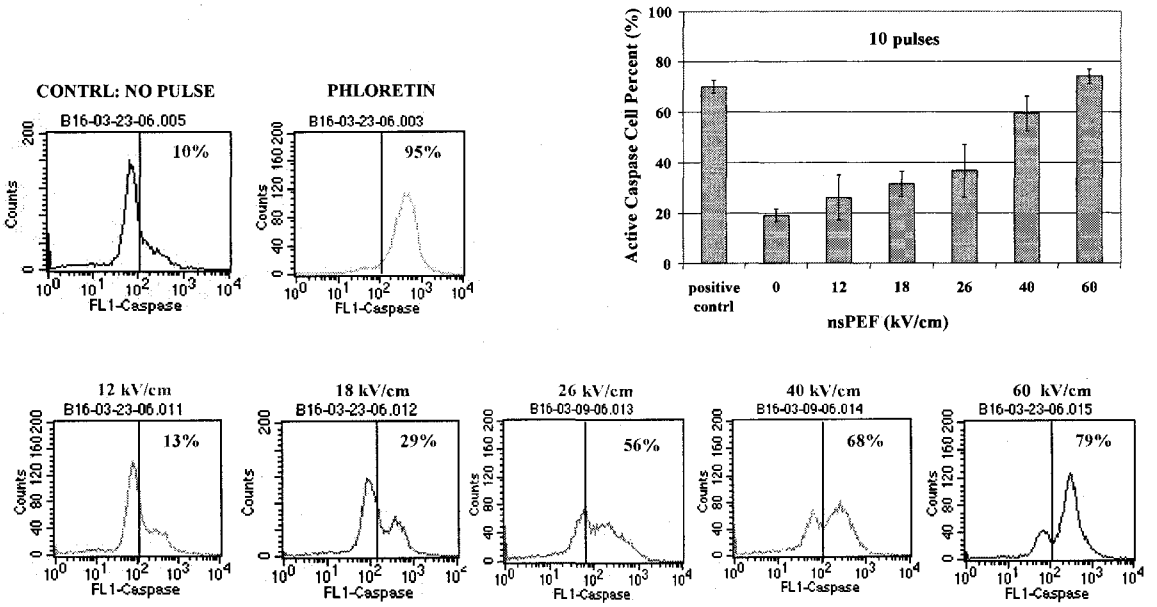

FIGURE 3 Nanosecond Pulsed Electric fields enhance caspase activation an electric field dependent increment. B16F10 melanoma cells exposed to nsPEFs previously mentioned or 200μM phloretin for one hour and caspase activity measured using a CaspACE™ FITC-VAD-FMK In Situ Marker 2 hours post pulse or n=3. Cells were analyzed by flow cytometry.

The uptake of Yo-Pro-1 is correlated with the activation of caspase in an electric field-dependent manner.

Short pulse durations with rapid rise-times have lesser effects on the plasma membrane and greater effects in intracellular membranes (Beebe et al, 2003b). Yo-Pro-1, is a unique indicator because it has the capability of identifying cells undergoing apoptosis while remaining impermeant to membrane integrity marker propidium iodide. This marker was used as an additional indicator for identifying apoptotic induction induced by nsPEFs correlated with caspase activation. The results indicate that nsPEFs induce apoptosis in B16F10 melanoma cells in an electric field dependent manner linked to caspase activation in the same manner (Figure 4).

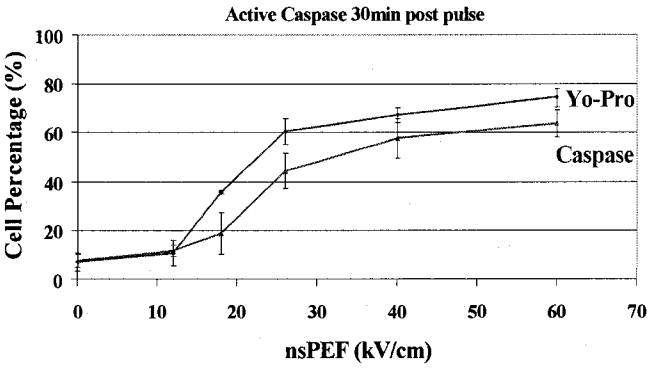

FIGURE 4 NsPEFs enhance Yo-Pro and caspase activation in an electric field dependent manner. B16F10 melanoma cells exposed to ten 300ns pulses ranging from 12-60kV/cm in the presence of Yo-Pro-1 (n=7). Cells were pre-incubated with FITC-VAD-fmk (n=7) for 20 minutes and washed. Cells were analyzed by flow cytometry 30min post pulse.

NsPEFs effects membrane permeability in a pulse number and electric field dependent manner.

Pore formation in the plasma membrane can attribute to cell death or lead to apoptosis induction. NsPEFs can have effects on the plasma membrane that may potentially be one contributing factor. Experimental data in Figure 5 suggest no significant effect on membrane permeability with one and three pulses at various electric fields. However, measured 30 minutes post-pulse, membrane permeability was not affected until cells were exposed to ten 300ns pulses at 40 or 60kV/cm when there was a 2-5-fold increase in fluorescence intensity in 10-30% of cells.

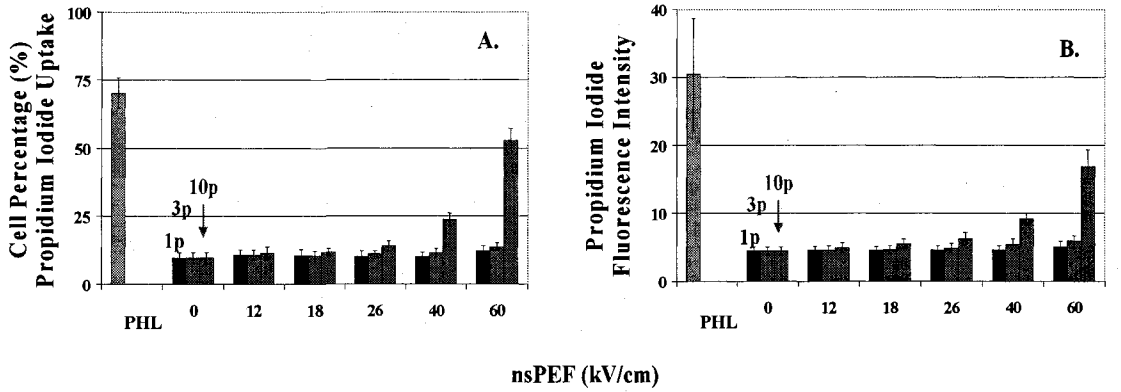

FIGURE 5 NsPEF effects membrane permeability determined by propidium iodide fluorescence intensity and cell percentage uptake. B16F10 melanoma cells were exposed to one, three and ten 300ns pulses ranging from 12-60kV/cm or 200μM phloretin (PHL) for 30min in the presence of propidium iodide. The percentage of cells exhibiting propidium iodide uptake in Panel A and the fluorescence intensity indicated by the geometric mean in Panel B were measured by flow cytometry 30min post pulse (n=3).

NsPEFs enhance membrane permeability in B16F10 melanoma cells in a time dependent manner.

In Figure 5, the effects nsPEFs have on membrane permeability in a pulse number and electric field dependent manner was examined. In order to identify the time at which the membrane permeability becomes comprised and the duration of pore opening, a sixty minute time course at one, three, five, ten, fifteen, thirty and sixty minute intervals was carried out. Data indicated in the first three minutes post pulse no significant difference in the percentage of cells (Figure 6B & 6D) or in the mean fluorescence (Figure 6A & 6C) were observed for PI uptake into the cells. However, there was a time-dependent increase in PI uptake reaching a maximum cell percentage and mean fluorescence 15 minutes post-pulse. Cells administered pulse in the presence of propidium iodide

illustrated a greater percentage cells that take up propidium iodide, (Figure 6D) than cells treated in the absence of propidium iodide (Figure 6B).

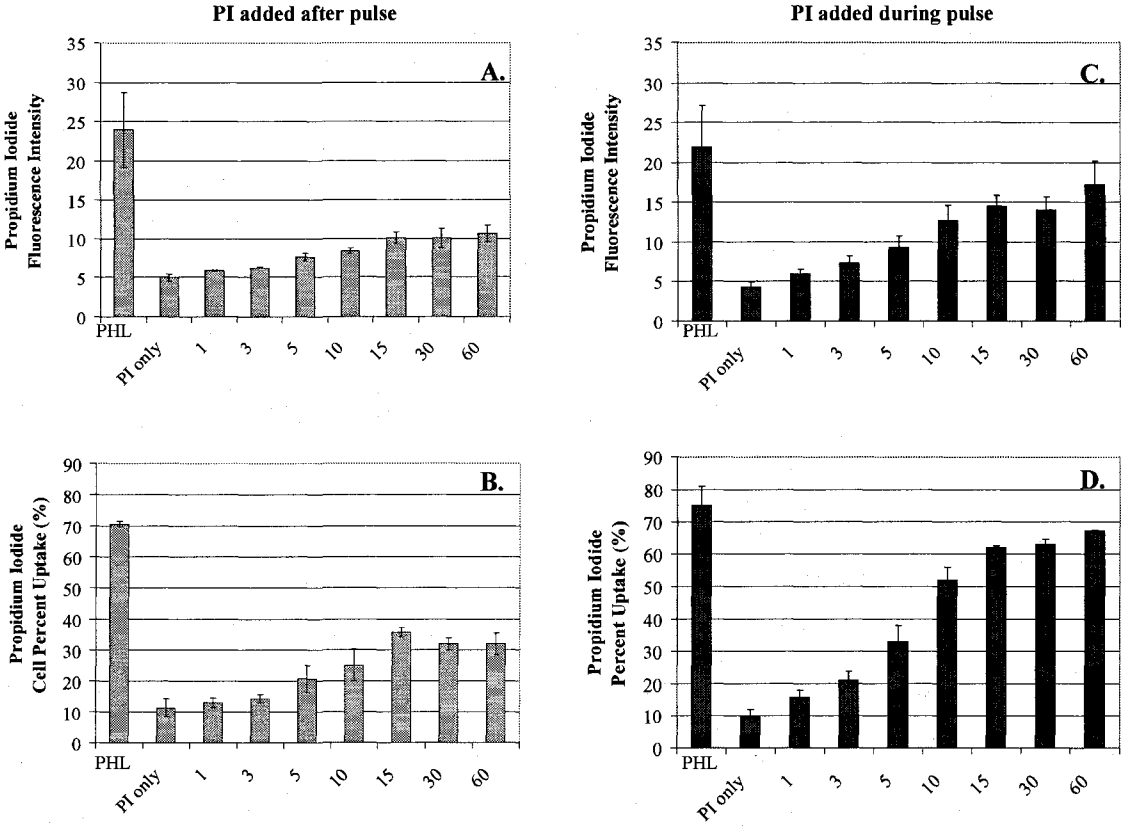

FIGURE 6 NsPEFs effects membrane permeability in an electric field and time dependent manner. B16F10 melanoma cells were exposed to ten 300ns pulses at 60kV/cm in the presence and absence of propidium iodide or 200μM phloretin (PHL) for one hour. The uptake was measured at times 1-60min by flow cytmetry (n=3). Panel A & C: propidium iodide uptake cell percentage and Panel B & D: propidium iodide fluorescence intensity geometric mean.

NsPEFs has minimal effects on phosphatidylserine(PS) externalization.

Another membrane marker used to examine nsPEF-induced effects was Annexin-V binding as a measure of PS externalization, which is a membrane indicator that identifies apoptotic cells. To determine effects of nsPEFs on membrane PS orientation, B16F10 melanoma cells were exposed to ten 300ns pulses and Annexin-V-FITC binding was determined by flow cytometry analysis 1hr post pulse. Data indicated that membrane integrity determined by PI uptake becomes comprised in less than 40% of cells in an electric field dependent manner (Figure 6B). However, under these conditions there are no significant effects of nsPEFs on PS externalization (Figure 7).

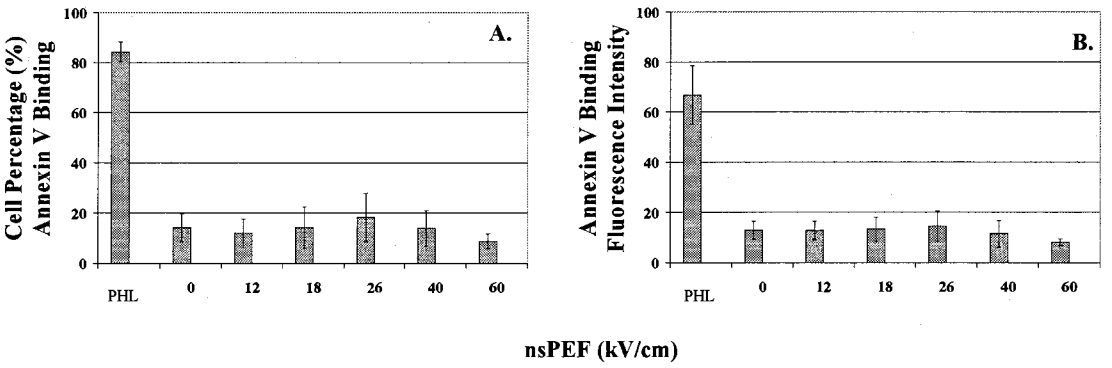

FIGURE 7 NsPEFs has minimal effects on phosphatidylserine externalization. B16F10 melanoma cells were exposed ten 300ns pulses at 60kV/cm or 200μM phloretin (PHL) for one hour and Annexin V (human recombinant FITC) added prior to flow cytometry analysis 1hr post pulse. Panel A demonstrates the percentage of cells that exhibit phosphatidylserine externalization via Annexin V binding and Panel B displays the fluorescence intensity indicated by the geometric mean (n=3).

NsPEF induce caspase activation in an electric field dependent manner independent of calcium mobilization.

Elevated intracellular calcium levels have been reported to induce caspase activation that leads to apoptosis (Mattson, 2003). In parallel experiments, calcium levels were determined in Fluo-3-AM loaded B16F10 cells (Figure 8A) and caspase levels were determined using FITC-VAD-FMK (Figure 8B) in the presence and absence of intracellular BAPTA-AM and extracellular EGTA. Ionomycin increased calcium 2-fold above basal levels, but no significant increases were observed in response to nsPEFs. The chelators decreased calcium 4-5-fold below basal levels to nearly undetectable quantities. When the chelators were present during nsPEF application, electric field-dependent increases in caspase activation was revealed with significant difference at 60kV/cm. This suggest that at 60kV/cm, calcium may potentially have an inhibit effect on caspase activation.

NsPEF disrupt cytoskeletal structure in B16F10 melanoma cells.

Caspase proteases are known to be involved in many morphological changes that initiate programmed cell death, one of which is alterations in the cytoskeleton structure (Slee et al., 2001; Elmore, 2007). Microscopy photographs, in Figure 9 illustrate the disruption of the F-actin cytoskeletal structure when ten 300ns pulses are applied at 60kV/cm 2 hrs post pulse, whereas cells not exposed to pulse conditions (0kV/cm) and anticancer agent phloretin maintains its F-actin cytoskeletal integrity; evident by number of nuclei that does not have F-actin associated with it in comparison to non-treated and phloretin treated cells.

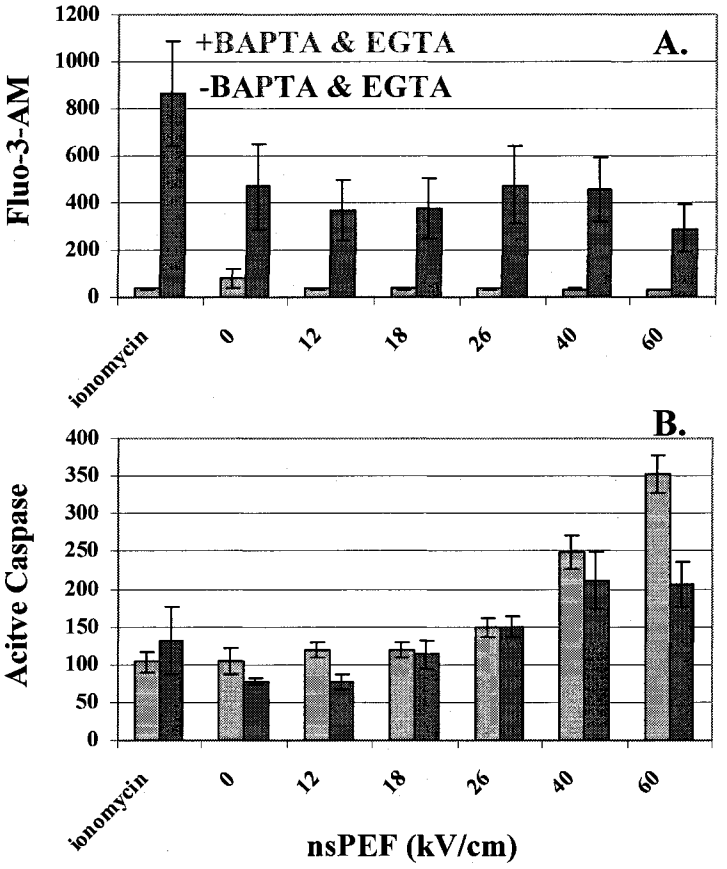

FIGURE 8 NsPEFs induce caspase activation independent of calcium mobilization. B16F10 melanoma cells were exposed ten 300ns pulses with electric fields ranging from 12-60kV/cm. The cells were pre-incubated in the presence and absence calcium chelators, BAPTA-AM and EGTA followed by incubation with calcium indicator, Fluo-3-AM and apoptotic marker FITC-VAD-FMK and analyzed via flow cytometry 30min post pulse in parallel experiments (n=4). Both Panel A and B represents the fluorescence intensity of the respective indicators Fluo3-AM and FITC-VAD-FMK indicated as the geometric mean. (positive control: 200μM phloretin). Panel B: +BAPTA-AM & EGTA Group significant difference with 60kV/cm vs. ionomycin and 0-40kV/cm & 40kV/cm vs. ionomycin & 0-18kV/cm; –BAPTA-AM & EGTA Group significant difference with 60kV/cm & 40kV/cm vs. 0 and 12kV/cm. Significant difference between +/-BAPTA-AM & EGTA Group at 60kV/cm ($P<0.05$).

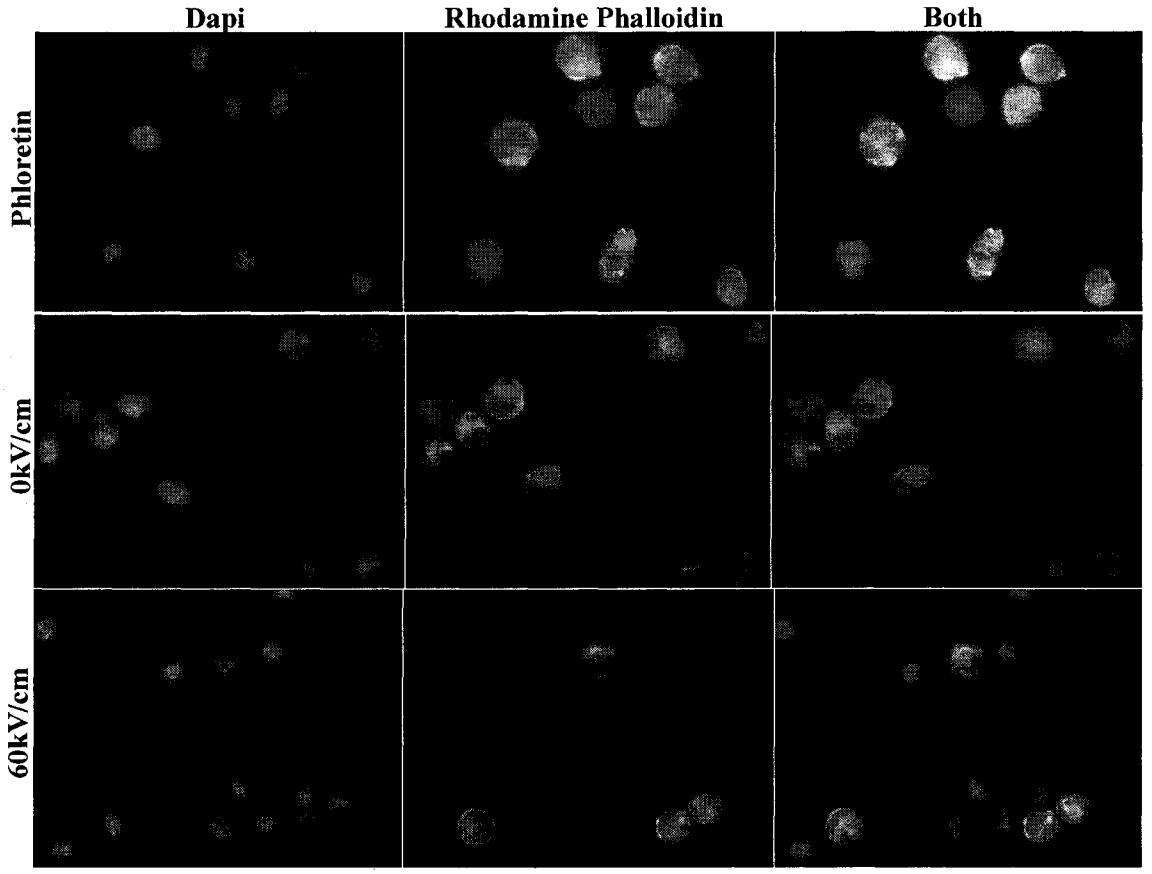

FIGURE 9 NsPEFs disrupt cytoskeleton structure. B16F10 melanoma cells were exposed to ten 300ns pulses at 60kV/cm or 200μM phloretin for two hours. Cells were fixed two hours post pulse, dried in an incubator at 37°C overnight, stained with rhodamine phalloidin, mounted with DAPI (blue:nucleus), cover slipped and viewed by an Olympus BX51 microscope and cells were captured with a DP70 camera.

NsPEF induce membrane blebbing.

Apoptosis is also characterized by bleb formation. Brightfield micrscopy images displayed irregular budding-like structures typical of blebbing when exposed to ten 300ns pulses at 60kV/cm two hours post pulse in comparison to 0kV/cm condition where cells reveal a more spherical structure.

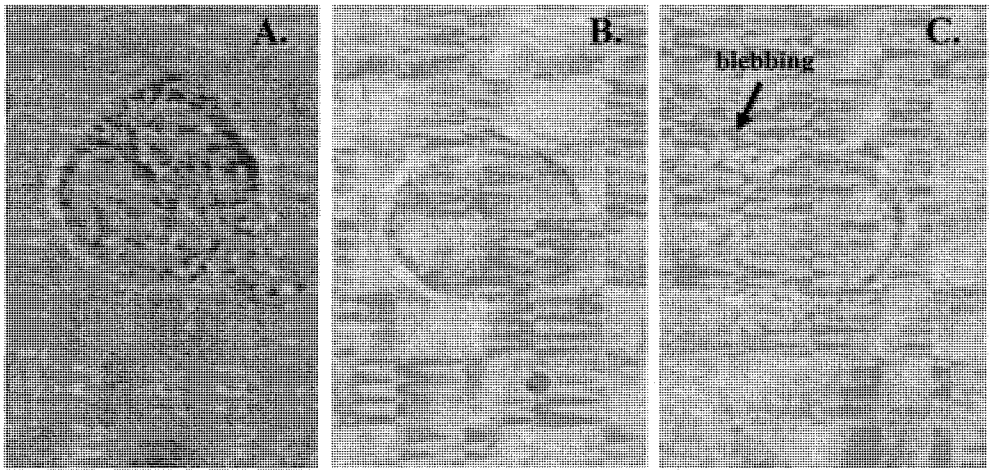

FIGURE 10 NsPEFs induce membrane blebbing. B16F10 melanoma cells were exposed to ten 300ns pulse at 60kV/cm or 200μM phloretin for two hours. Cells were fixed two hours post pulse, dried in incubator at 37°C overnight, stained, mounted with DAPI (blue:nucleus), cover slipped and viewed by an Olympus BX51 microscope and brightfield images captured with a DP70 camera. (A). Cell treated with phloretin (B). 0kV/cm (C). 60kV/cm.

Discussion

Previous *in vitro* and *in vivo* studies suggest nsPEFs initiate apoptosis induction. The application of these ultrashort pulse durations has been illustrated to activate caspases, intracellular proteases essential in the apoptotic process, phosphatidylserine externalization, enhanced pro-apoptotic protein Bax activity and DNA fragmentation all associated with apoptosis (Beebe et al, 2003a; Vernier et al., 2003; Stacey et al., 2003; Vernier et al., 2004; Hall et al., 2007). Initial research in mouse B10-2 fibrosacoma tumors reported nsPEFs initiate caspase activation and DNA fragmentation by TUNEL *ex vivo* while tumor reduction was observed *in vivo* (Beebe et al., 2002). Recent studies in B16F10 mouse melanoma cells showed tumor regression though precise mechanism involved with tumor shrinkage was not well defined (Nuccitelli et al., 2006). The results paved the way to further investigate cell death mechanism *in vitro* associated with nsPEFs applications.

Here the administration of 300ns pulses ranging from 12kV/cm-60kV/cm were applied to B16F10 mouse melanoma cells. As detected in earlier studies, the administration of nsPEF treatments induced caspase activity in B16F10 melanoma cells. The data reveal that the application of 300ns pulses ranging from 12kV/cm-60kV/cm enhances caspase activity in an electric field and pulse number dependent manner measured two hours post treatment. Neither one nor three pulses were sufficient to stimulate an electric field dependent increase in caspase activity nor to result in a decrease in cell number. However, ten pulses were sufficient to overcome threshold that recruits a greater population of cells for caspase activity in an electric field dependent manner. For the first time electric field and pulse number dependent increase in caspase

activation was correlated with cell survivability twenty four hours post pulse. Since caspases are known to be involved in the eventual death of the cell, data suggest that nsPEFs mediate a caspase-dependent apoptosis linked with cell death. In addition, results using Yo-Pro-1 as an apoptotic indicator displayed an electric field dependent increase correlated with caspase activity. The results further support a caspase-dependent apoptosis induced by nsPEFs.

Another distinct feature of apoptosis is characterized by effects on the plasma membrane. Pores created in the plasma membrane can have a significant impact on cell viability. Convectional electroporation, which was generally designed to maximize cell survival, deliver long electrical pulses ranging in durations from milliseconds to microseconds that form large pores in the plasma membrane that significantly enhance the flow of ions and molecules across the plasma membrane (Weaver, 1995). This application has been shown to be advantageous in procedures such as DNA transfection or transformation (Nickoloff, 1995), cancer tumor electrochemotherapy (Dev and Hofmann, 1994), gene therapy (Tjelle et al., 2006) and trans-dermal drug delivery (Prausnitz *et. al.,* 1993). On the other hand, formation of large pores allows non-specific molecules to move in and out of the cell that could lead to improper cell function and cell death (Weaver, 1995). Along these lines, specific damage to cells using irreversible electroporation is now in use as a means to eliminate cancer.

In contrast, nanosecond pulses have much shorter pulse durations and display differential effects on the plasma membrane, which may not always be observable using propidium iodide uptake, and alterations in phosphatidylserine externalization (PS). Based on *in silico* studies by Gowrishankar et al. (2006) and Smith and Weaver (2008),

nsPEFs result in supra-electroporation of the plasma membrane and organelle membranes with high densities of nanopores. These nanopores are on the order of 1 nm which is about the size of propidium iodide. The measurement of membrane permeability by propidium iodide uptake suggests nsPEFs induce a biological effect on the plasma membrane and not a direct effect on plasma membrane (Beebe et al., 2003; Chen et al., 2004). Similar observations were revealed in the uptake of propidium iodide in B16F10 melanoma cells. Initial results demonstrated no effect on the plasma membrane with one and three pulses whereas ten pulses illustrated an electric field and pulse number dependent increase for propidium iodide uptake. Furthermore, maximal plasma membrane permeability was observed by propidium iodide uptake within fifteen minutes of sixty minute time course post nsPEF application in the absence and presence of propidium iodide. Approximately thirty percent more cells pulsed in the presence of propidium iodide took up propidium iodide. It is likely that nsPEFs induce nanopore formation in B16F10 membranes that are too small to allow propidium iodide uptake. Thus, the observed PI uptake that is time dependent in a population of cells is likely due to the later stages of apoptosis where the plasma membrane begins to loose integrity.

A different marker to evaluate changes at the plasma membrane uses Annexin V that measures externalization of phosphatidylserine phospholipids exposed during apoptosis. NsPEF initiate alterations in PS as shown in Jurkats (Beebe et al., 2003a; Vernier et al., 2004) that differs in B16F10 melanoma cells. Minimal PS externalization was observed regardless of the nsPEF condition in B16F10 melanoma cells. However, B16 cells can externalize PS evident by phloretin treated cells which suggest that 300ns PEFs condition does not externalize PS. Thus, the absence of PS externalization appears

to be unique to nsPEF-induce caspase dependent apoptosis in comparison to phloretin-induced apoptosis and caspase dependent apoptosis demonstrated in Jurkats and HCT 116 cells (Beebe et al., 2003a; Hall et al., 2005).

Further evaluation of morphological changes indicative of apoptosis that involve the plasma membrane additionally strengthen nsPEF involvement in this form of programmed cell death. The loss of the active cytoskeleton and the presence of membrane blebbing are two such changes that occur during apoptosis and are likely related. The activation of caspases may mediate membrane blebbing since actin is a target for caspase proteolysis (Coleman et al., 2001). Here membrane blebbing was visible by bulging out pockets of plasma membrane under 300ns 60kV/cm pulse conditions.

The release of calcium intracellular signaling molecule from intracellular organelles plays a key role in many physiological and cellular functions such as apoptosis induction (Zhang et al., 2006). The mobilization of intracellular calcium has been linked to caspase activation and DNA fragmentation by Ca^{2+}-dependent endonucleases (Mattson, 2003; Bortner et al., 1995). However, studies have demonstrated apoptosis induction independent of Ca^{2+} fluctuations (Grubb 2001). According to study conducted, no differences in the levels of intracellular calcium release were observed under nsPEF conditions ranging from 12-60kV/cm though calcium mobilization from intracellular induced by nanosecond pulse conditions has been reported (Vernier et al., 2003; White et al., 2004; Zhang et al., 2008). The data suggest an electric field dependent increase in caspase activation independent of intracellular calcium release. However, significant

differences between BAPTA-AM +/- group at 60kV/cm indicate that at ten 300ns pulses at 60kV/cm calcium may potentially have an inhibitory role in caspase activation.

Several characteristic morphological changes occur during apoptosis. The cytoskeleton structure is composed of three kinds of filaments, actin filaments (microfilaments), intermediate filaments, and microtubules that provide shape and structure to the cell. Caspases are known to be responsible for cellular breakdown and mediate actin disruption (Watanabe and Akaike, 1999; Slee et al., 2001). *In vitro* study in B16F10 melanoma cells illustrated actin cytoskeleton breakdown induced by nsPEFS similar to previous report in HCT 116 carcinoma cells (Hall et al., 2007). At nsPEF conditions of 300ns 60kV/cm and ten pulses, the majority of cells lost their F-actin cytoskeletal integrity within two hours of treatment. Different cytoskeletal effects were observed with phloretin treated cells which maintained their integrity within two hours of treatment. This indicates that nsPEF treated cells are in a later stage of apoptosis in comparison to phloretin treated cells. Also caspases may contribute to this breakdown as indicated in previous observations.

Caspase activation occurs within the first hour after application of nsPEFs. Apoptosis takes place in three phases, initiation, a latent phase and an execution phase. For nsPEFs, it appears that the initiation phase and the latent phase are relatively short since caspase activation marks the execution phase within the first hour. This suggests that nsPEFs induce an insult that is rapidly perceived as a clear and present danger that cannot co-exist with survival and the apoptosis program is quickly initiated.

Overall, data illustrates that nsPEFs induce apoptosis characterized by a rapid electric field dependent increase in calcium-independent caspase activation in the absence

of PI uptake or PS externalization, and a disruption of F-actin cytoskeletal structure with membrane blebbing. This paves the way in the next chapter to further examine specific apoptotic pathway(s) associated with apoptotic induction in B16F10 melanoma cells.

CHAPTER III

NANOSECOND PULSED ELECTRIC FIELDS INDUCE APOPTOSIS INDEPENDENT OF MITOCHONDRIA IN B16F10 MELANOMA CELLS *IN VITRO*

INTRODUCTION

Apoptosis mechanisms are complex. The fate of the cells depends on a complex set of sensors and positive and negative regulators whose balance will determine whether apoptosis is initiated or not. Unfortunately, no linearly defined apoptosis death pathways exists, but for convenience they are frequently grouped into two main categories including an intrinsic mechanism, which is linked to the mitochondria, and an extrinsic mechanism, which is linked to death receptors in the plasma membrane. The intrinsic pathway is dependent upon the mitochondrial release of cytochrome c activation in response to intracellular stresses such as DNA damage, serum deprivation, and stress to endoplasmic reticulum (Adams, 2003; Aoki 1996). The Bcl-2 family of proteins consists of pro- and anti- apoptotic members associated with the mitochondria's role in apoptosis. The sensitivity of cells to apoptotic stimuli depend on the balance between pro- and anti- apoptotic proteins. The increase in pro-apoptotic protein levels such as Bax disrupts the normal function of the anti-apoptotic Bcl-2 proteins leading to pore formation in the mitochondria. The alteration in the mitochondrial membrane potential leads to release of mitochondrial protein cytochrome c, which binds to cytosolic proteins, APAF-1 and pro-caspase -9 to form the apoptosome, which proteolytically activates

caspases -9 leading to activation of executioner caspases -3, -6 and -7 and ultimately cell death (Albert, 2002).

Additionally, the mitochondria release other mitochondrial proteins in similar manner to cytochrome c. The release of Smac/DIABLO interacts with the IAP family of apoptosis inhibitory proteins to neutralize their anti-apoptotic activity to induce cell death (Hengartner, 2000). Also mitochondrial release of apoptosis inducing factor (AIF) initiates DNA fragmentation and nuclear morphological changes such as chromatin condensation that is associated with apoptotic cell death (Jacobson and McCarthy, 2002).

The extrinsic pathway consists of a mitochondrial-independent (type I) and mitochondrial-dependent (type II) apoptotic induction dependent on signaling through the plasma membrane. The extrinsic type I pathway is initiated by interaction between the death receptors (Fas, TNF, TRAIL) and death ligands. As a result of this interaction death adaptor proteins (FADD, TRADD) are recruited to the cytosolic domains of the receptors in the plasma membrane. The death inducing signaling complex (DISC) forms with the adaptor proteins and pro-caspase 8, which leads to activation of caspase -8 and then activation of caspase -3. The extrinsic mitochondria-dependent type II pathway, utilizes death receptors however, caspase -8 cleaves pro-apoptotic protein, Bid. The truncated Bid (t-Bid) acts on the mitochondria to cause cytochrome c release, thus activating caspase -9 in the apoptosome and then -3. Thus t-Bid connects the extrinsic pathway to the intrinsic pathway.

As mentioned previously, reports have suggested that nsPEFs initiate caspase-dependent apoptotic cell death measured by various apoptotic indicators. However the apoptotic mechanisms and pathway(s) require further definition. Thus far only one study

performed in Jurkats T lymphocytes has pointed toward the involvement of a mitochondria dependent apoptotic pathway observed by cytochrome c release from mitochondria and caspase activation (Beebe et al., 2003). Based on experimental data from Chapter 2 that nsPEFs induce apoptosis in B16F10 melanoma cells *in vitro* leads to further assessment of specific apoptotic pathway(s) through which these events take place. The examination of the intracellular release of mitochondrial proteins, cytochrome c, Smac/DIABLO and AIF and the identification of specific initiator caspases -8 and-9 and executioner caspases -3, -6, and -7 would clarify whether an intrinsic or extrinsic type II mitochondrial dependent or extrinsic type I mitochondrial independent apoptotic pathway is involved in this cell death process.

The results showed no mitochondrial involvement in the induction of apoptosis induce by nsPEFs as revealed by the absence of release of mitochondrial proteins cytochrome c, Smac/DIABLO and AIF. Though initiator caspase -8 and executioner caspases -3, -6 and -7 were detected, interestingly caspase -9 activation was also apparent. The known mechanism for caspase -9 activation requires apoptosome formation through mitochondria release of cytochrome c and APAF-1 was not identified in experiment. Data suggest that nsPEFs initiate a mitochondrial independent apoptosis that may induce cell death signaling that involves the plasma membrane. Although no mitochondrial activity was observed, nsPEFs trigger caspase -9 activation via direct cleavage or by another unknown mechanism not involving formation of the apoptosome.

EXPERIMENTAL PROCEDURE

Cell Culture

Mouse melanoma cells (B16F10) used in study was obtained from ATCC (Manassas, VA). B16 cells grown in Dulbecco's Modified Eagle's Medium with L-glutamine (ATCC; VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals; Nocross, GA), 1% L-glutamine and 1% penicillin streptomycin (Mediatech Cellgro; VA) and placed in incubator at 37°C with 5% CO_2.

Cell Preparation

B16F10 cells for treatment were detached from cultured flask when cells are about 80-90% confluent. This involves removal of media from flask, a rinse with Hank's Balanced Salt Solution 1X (Mediatech Cellgro;VA), trypsin EDTA 1X 0.25%trypsin/2.21mM EDTA in HBSS addition (Mediatech Cellgro;VA) and DMEM culture medium addition. B16F10 cell preparation prior to pulse involves centrifugation at 1000rpm and addition of Dulbecco's Phosphate Buffered Saline (DPBS) solution which contains 0.5mM $CaCl_2.2H_2O$, 4.2mM KCl, KH_2PO_4, 0.5mM $MgCl_2.6H_2O$, 153mM NaCl, 9.5mM $Na_2HPO_4.7H_2O$ and 2.5mM glucose, pH 7.4. Cell count was performed using a hemacytometer, centrifugation at 1000 rpm and DPBS solution addition based on $7.7x10^6$cells/mL.

Administration of nsPEF

Trypsinized B16 cells $7.7x10^6$cells/mL are exposed to nsPEF conditions at a concentration of $1x10^6$cells/130μL placed in 0.1cm gene pulser cuvettes (Bio-Rad Laboratories; Hercules, CA). Rectangular high voltage pulses of 300 ns duration and amplitudes of 1.2-6.0 kV are applied by transmission line type pulse generator to the

plane parallel electrodes in the cuvette. Representative pulse shapes are shown in Figure 1. The 10-Ohm impedance of the pulse generator is matched to the resistance of the exposed sample. With increasing voltage, the rise time of the pulses increases slightly from 15.3 to 19.1 ns. The applied voltages correspond to homogeneous electric fields of 12-60 kV/cm in the cuvette.

Measurement of Cytochrome C, Smac/DIABLO and AIF Release From the Mitochondria

B16F10 melanoma cells were prepared and exposed to nsPEFs as previously mentioned, incubated with monoclonal antibodies for cytochrome c (BD Pharmingen; San Jose, CA), SMAC/DIABLO (Calbiochem; Gibbstown, NJ) and AIF (Santa Cruz Biotechnology; Santa Cruz, CA) for one hour. Cells were washed in 1mL of modified phosphate buffer followed by one hour incubation with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen; Oregon) and washed in 1X PBS. Fifteen thousand cells were acquired and analyzed by a Becton-Dickinson FACSCalibur flow cytometer. Data analysis was carried out by Cell Quest Pro software for fluorescence intensity measurement.

Evaluation of Active Caspases -8, -9, -6, -3 & -7 by Flow Cytometry

Cells were prepared and exposed to nsPEF conditions as previously mentioned. Live cells were according to manufacturer's instructions from Carboxyfluorescien FLICA Kit (Immunochemistry Technology LLC; Minnesota) specific for caspases -8, -9 and -6 and Sulforhodamine FLICA Kit (Immunochemistry Technology LLC; Minnesota) specific for caspases -3 and -7. Ten thousand cells were acquired using BD FACS Aria and analyzed by BD FACSDiva software.

Evaluation of Active Caspases -8, -9, -6, -3 & -7 by Fluorescence Microscopy

Cells were prepared and exposed to nsPEF conditions as previously mentioned. Cells were fixed according to manufacturer's instructions from Carboxyfluorescien FLICA Kits (Immunochemistry Technology LLC; Minnesota) specific for caspases -8, -9 and -6 and Sulforhodamine FLICA Kit specific for caspase -3 and -7 (Immunochemistry Technology LLC; Minnesota). Cells were dried on slides overnight in the incubator at 37°C with 5% CO_2, cover slipped and mounted with 4 ,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for nuclei staining. Cells were viewed by an Olympus BX51 microscope and images captured by DP70 camera.

RESULTS

Cytochrome C release is undetectable in B16F10 melanoma cells post pulse.

In order to determine whether nsPEFs induce apoptosis through an intrinsic or extrinsic pathway that involves the mitochondria, cytochrome c release was examined. Figure 11 shows that cytochrome c remains undetected for 5 hours post pulse. A 2-fold increase in about 50% of the cells was observed at 7 hours in comparison to earlier time point. In contrast, phloretin induced a time dependent increase in cytochrome c release indicating that B16F10 cells can function through a mitochondria dependent mechanism, but not in response to nsPEFs.

Mitochondrial proteins, Smac/DIABLO and AIF release undetectable in B16F10 melanoma cells upto to 3hrs post pulse.

In addition to investigating the mitochondria via cytochrome c release, other mitochondrial proteins, Smac/DIABLO and AIF that are released during apoptotic induction were assessed. Figure 12 illustrates no release of mitochondrial proteins

Smac/DIABLO or AIF. In contrast, phloretin treated cells exhibited an approximate 4 to 5 and 2.5 to 4 fold increase in fluorescence intensity for Smac/DIABLO and AIF respectively within 3 hours post pulse. Thus, release of these pro-apoptotic proteins can function in B16F10 cells, but not in response to nsPEFs.

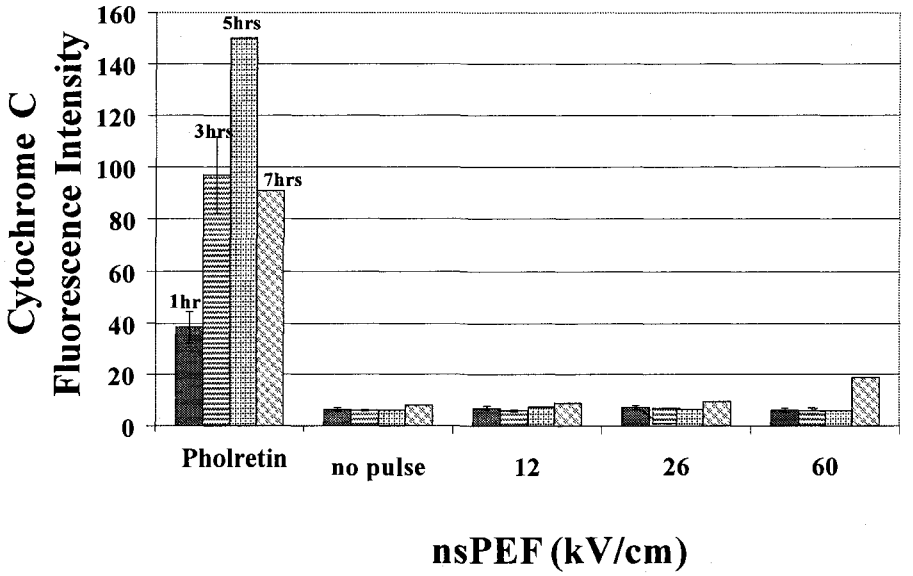

FIGURE 11 No detection of cytochrome c release in B16F10 melanoma cells exposed to nsPEFs 5hrs post pulse. B16F10 melanoma cells were exposed to ten 300ns pulses at 12, 26, and 60kV/cm or 200μM phloretin for one hour, incubated with monoclonal anti-cytochrome c antibody, followed by incubation with Alexa Fuor 488 goat anti-mouse antibody. Cytochrome c fluorescence intensity is indicated as the geometric mean 1hr (n=3), 3hrs (n=3), 5hrs (n=1) and 7hrs (n=1).

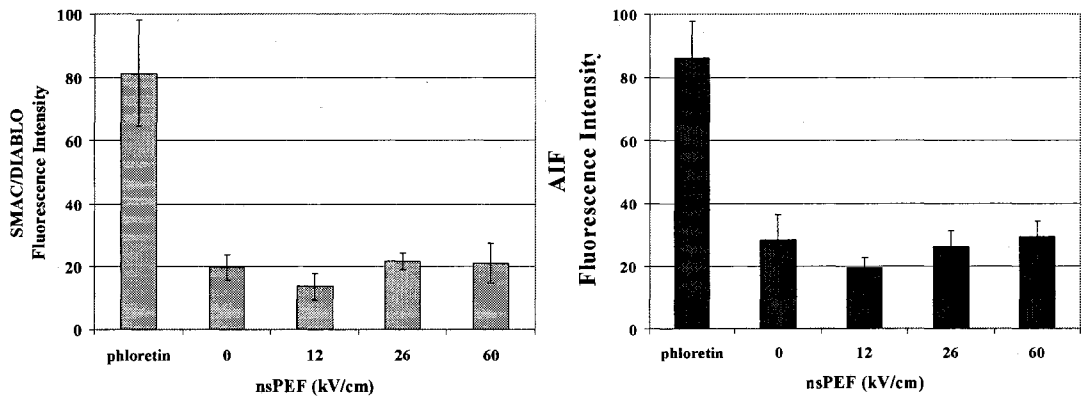

FIGURE 12 NsPEFs have minimal effects on the release of mitochondrial proteins SMAC/DIABLO and AIF. B16F10 melanoma cells were exposed to ten 300ns pulses ranging from 12 -60kV/cm or 200μM phloretin for one hour, incubated with monoclonal SMAC/DIABLO and AIF antibodies, followed by incubation with Alexa Fluor 488 goat anti-mouse antibody. SMAC/DIABLO and AIF fluorescence intensity is indicated as the geometric mean at 3hrs (n=4 & 5 respectively).

NsPEFs induce activation of initiator caspases -8 and -9.

The flow cytometry data represented in Figure 13 demonstrates an electric dependent increase in both the activation of initiator caspases -8 and -9 with approximately 60% of cell population demonstrating activity at 60kV/cm. Similar observations were viewed in fluorescent microscopy images where over 60% cell population demonstrated both active caspase -8 and -9 indicated by intracellular green fluorescence in Figure 14 and 15 respectively in response to at electric field condition 60kV/cm. Activation of initiator caspases -8 and -9 were observed in phloretin-treated cells.

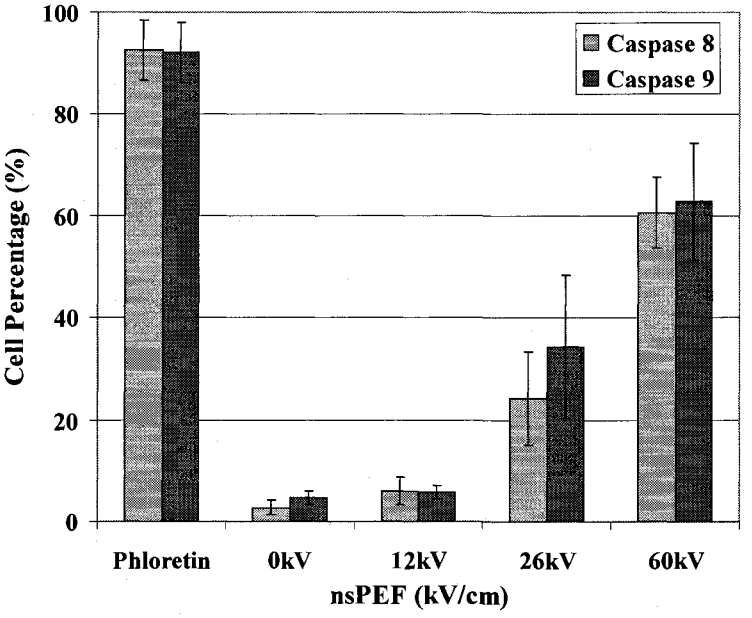

FIGURE 13 NsPEF induce activation of initiator caspases -8 and -9 in an electric-field dependent manner. Cells were exposed to ten pulses ranging from 12-60kV/cm or 200μM phloretin for one hour and percentage of cells active for caspases -8 and -9 measured using a FAM-LETD-FMK and FAM-LEHD-FMK *In Situ* marker, representatively. Cells analyzed by flow cytometry 2 hours post pulse (n=5 except 0kV/cm).

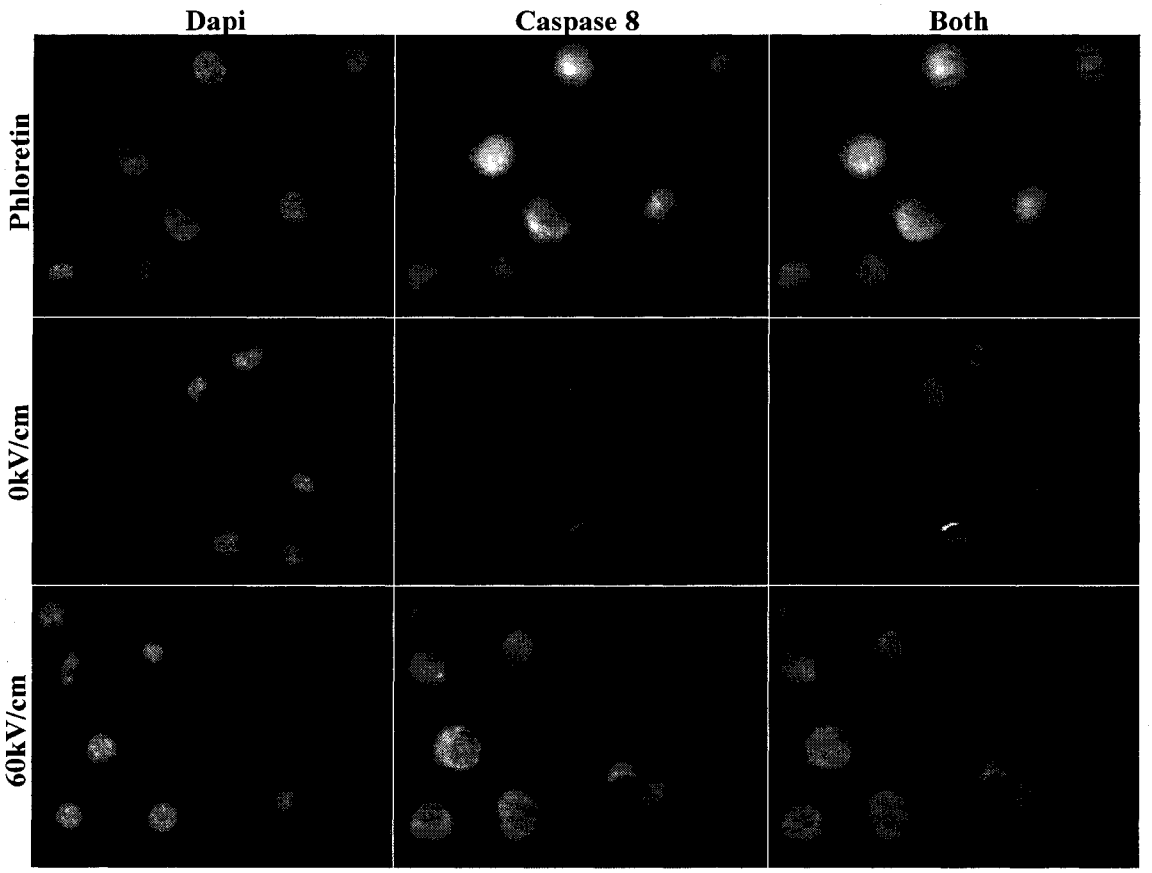

FIGURE 14 NsPEFs induce activation of initiator caspase -8. B16F10 melanoma cell were treated with ten 300ns pulses at 60kV/cm or 200μM phloretin for two hours, incubated with FAM-LETD – FMK indicator, fixed two hours post pulse, dried in an incubator at 37°C overnight, mounted with DAPI (stains nucleus), cover slipped and viewed by an Olympus BX 51 microscope and cells were captured with DP70 camera.

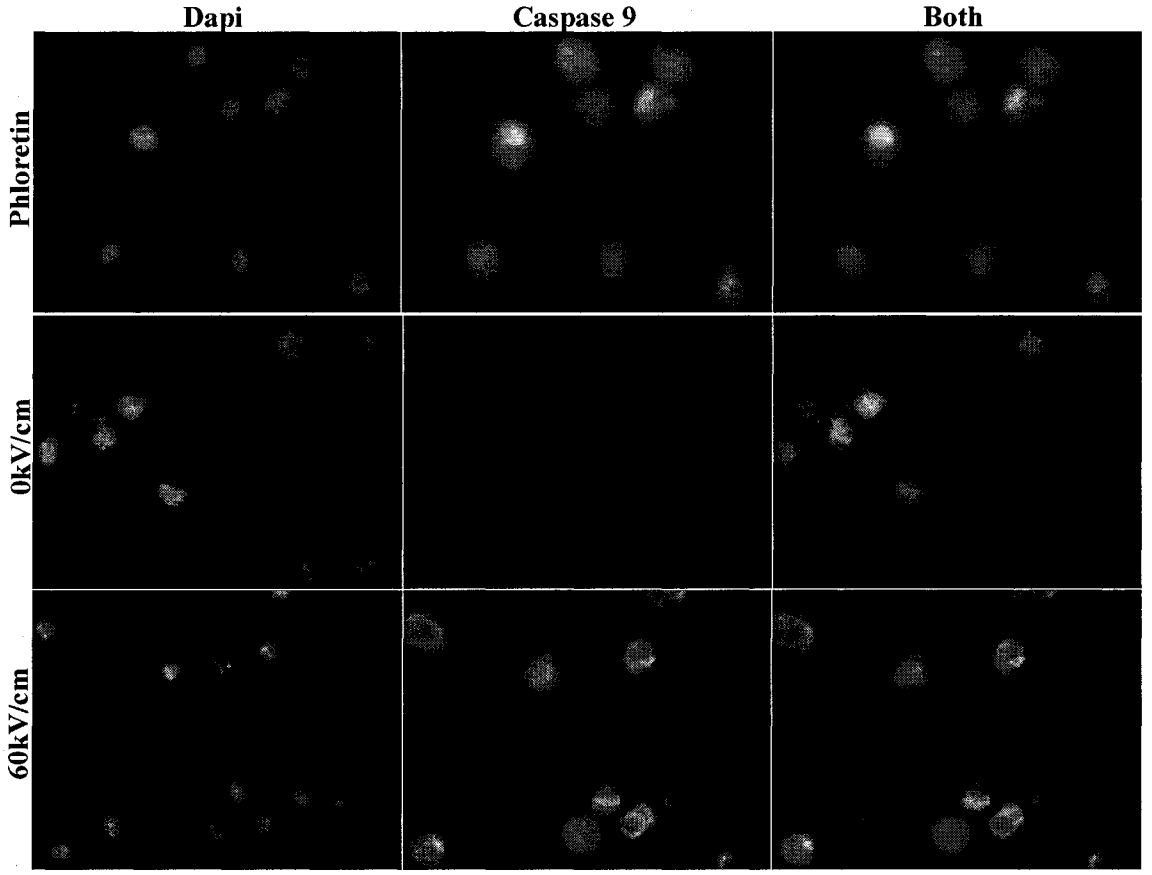

FIGURE 15 NsPEFs induce activation of initiator caspase -9. B16F10 melanoma cell were treated with ten 300ns pulses at 60kV/cm or 200μM phloretin for two hours, incubated with FAM-LEHD – FMK indicator, fixed two hours post pulse, dried in an incubator at 37°C overnight, mounted with DAPI (stains nucleus), cover slipped and viewed by an Olympus BX 51 microscope and cells were captured with DP70 camera.

NsPEFs induce activation of executioner caspases -3, -6 and -7

The data represented in Figure 16 demonstrates an electric-field dependent increase in the activation of executioner caspases, -3, -6 and -7 with approximately 60% of cell population expressing activity at 60kV/cm. Similar results were displayed four hours post pulse (data not shown). Comparable observations were viewed in fluorescent microscopy images where about 80% and 70% of cell population demonstrated both active caspase -6 and -3/-7 indicated by intracellular green and red fluorescence in Figure 16 and 17 respectively at electric field condition 60kV/cm. Active executioner caspases -6 and -3/-7 were observed in phloretin-treated cells.

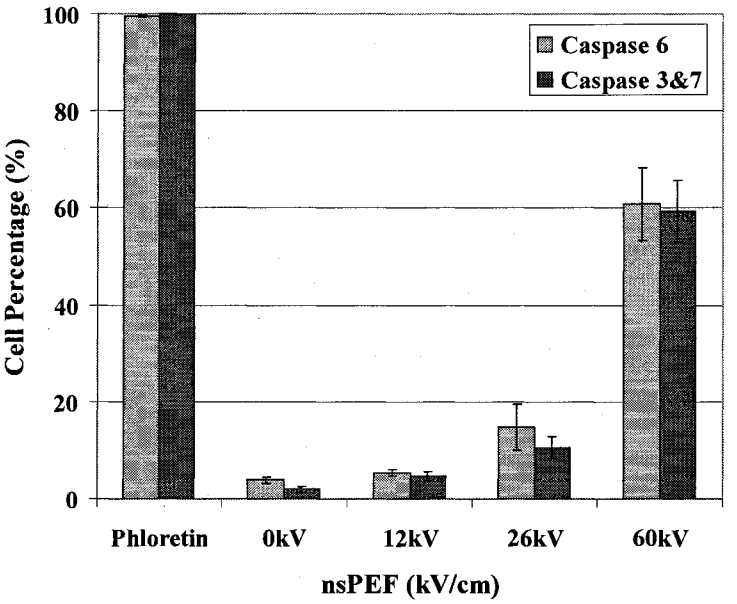

FIGURE 16 NsPEF induce activation of executioner caspases -3, -6 & -7 in an electric-field dependent manner. Cells were exposed to ten pulses ranging from 12-60kV/cm or 200μM phloretin for one hour and percentage of cells active for caspases -6 and -3 & -7 measured using a FAM-VEID-FMK and SR-DEVD-FMK *In Situ* marker, representatively. Cells analyzed by flow cytometry 2 hours post pulse (n=4).

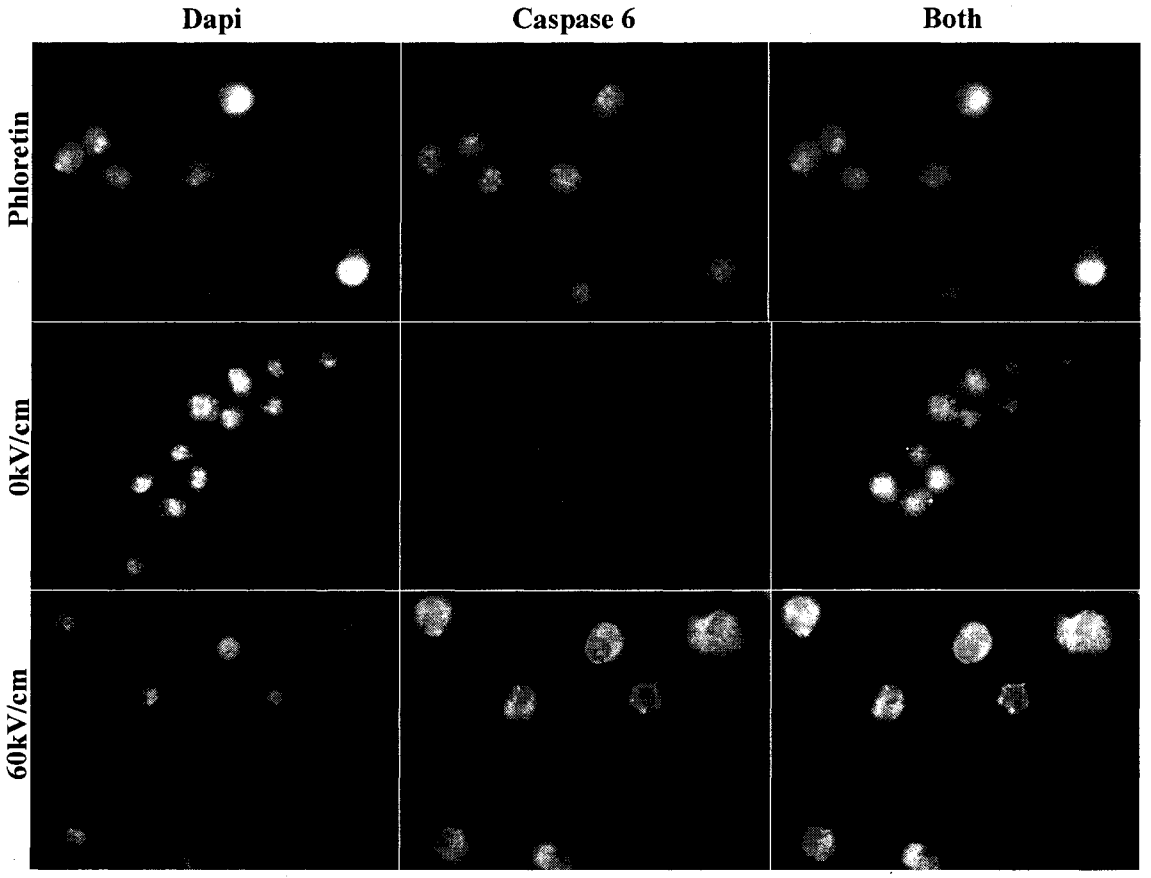

FIGURE 17 NsPEFs induce activation of executioner caspases -6. B16F10 melanoma cell were treated with ten 300ns pulses at 60kV/cm or 200μM phloretin for one hour, incubated with FAM-VEID–FMK indicator, fixed two hours post pulse, dried in an incubator at 37°C overnight, mounted with DAPI (stains nucleus), cover slipped and viewed by an Olympus BX 51 microscope and cells were captured with DP70 camera.

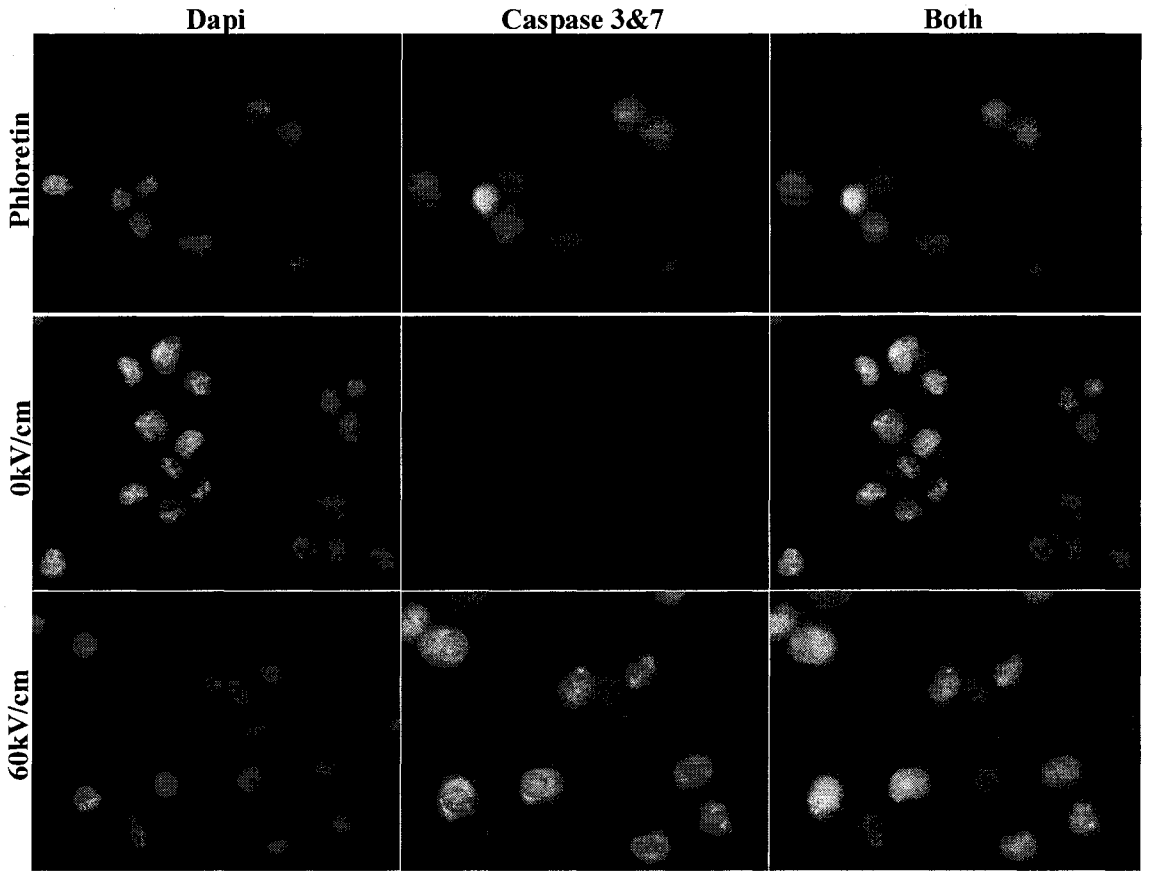

FIGURE 18 NsPEFs induce activation of executioner caspases -3 & -7. B16F10 melanoma cell were treated with ten 300ns pulses at 60kV/cm or 200μM phloretin for two hours, incubated with SR-DEVD –FMK indicator, fixed two hours post pulse, dried in an incubator at 37°C overnight, mounted with DAPI (stains nucleus), cover slipped and viewed by an Olympus BX 51 microscope and cells were captured with DP70 camera.

DISCUSSION

Numerous studies have recognized nsPEF-induced apoptotic cell death distinguished by caspase activation, PS externalization, propidium iodide uptake, DNA fragmentation, cytochrome c release and pro-apoptotic protein, BAX (Beebe et al, 2003a; Stacey et al., 2003; Vernier et al., 2004; Hall et al., 2007). The experimental data in Chapter 2 demonstrated similarities in apoptotic induction by nsPEFs in B16F10 melanoma cells by caspase activation although no PS externalization was identified. To further strengthen apoptosis induction I illustrated morphological changes of plasma membrane blebbing and cytoskeleton disruption, which were not shown previously in response to nsPEFs. Only one study suggested that nsPEFs may potentially induce cell death through a mitochondrial dependent apoptosis mechanism identified by mitochondria release of cytochrome c in Jurkat cells (Beebe et al., 2003). However, this study does not specify whether apoptotic induction by nsPEFs occurred through a mitochondrial dependent intrinsic pathway or a type II extrinsic apoptotic pathway (Beebe et al., 2003a).

Intracellular proteins are associated with the classification of specific apoptotic pathways. The mitochondria function plays an essential part in defining the intrinsic or extrinsic type II apoptotic pathway through release of mitochondrial proteins necessary to complete execution of cell death. The mitochondria release of cytochrome c, more importantly is an essential protein required for the activation of initiator and executioner caspases through the mitochondria-dependent pathways. *In vitro* evaluation in B16F10 melanoma cells displayed no detection of cytochrome c release from the mitochondria up to seven hours post nsPEF application. However, at seven hours an approximate 2.5 fold

increase in comparison to five hours was observed. The event is far too late for the involvement in caspase activation observed in less than one hour post pulse. In addition to the absence of mitochondria cytochrome c release, similar results were revealed with other mitochondrial proteins, Smac/DIABLO and AIF, indicating that nsPEFs initiate a mitochondria independent apoptosis. In contrast B16F10 melanoma cells treated with anticancer agent phloretin demonstrated release of mitochondrial protein cytochrome c indicating that a mitochondrial dependent apoptotic pathway can be activated in B16F10 cells, but not by nsPEFs. Also the observed release of additional mitochondrial proteins Smac/DIABLO and AIF supports the mitochondria involvement in apoptotic cell death mechanism in response to phloretin, but not by nsPEFs. This eliminates the possibility that B16F10 melanoma cells are incapable of releasing mitochondrial proteins and suggest differential effects in apoptotic induction between phloretin and nsPEFs.

Earlier reports have showed that pulse electric fields stimulate a caspase-dependent apoptosis. However specific caspase(s) involvement in the process was not investigated (Beebe et al., 2003a). In this study initiator caspases -8 and -9 were investigated to further determine a mitochondria dependent or independent apoptotic pathway. The data represented demonstrates an electric field dependent increase in both initiator caspases -8 and -9 despite the absence of cytochrome c release from the mitochondria, which is the only known mechanism for caspase -9 activation. In addition, data was further supported by the expression of active caspase -8 and -9 in fluorescent microscopy images. The activation of caspase -9 along with the absence of mitochondria release of cytochrome c implies the possibility that there maybe some other mechanism besides apoptosome formation with cytochrome c and APAF-1 for activation of caspase -

9 by nsPEFs. Data illustrated that nsPEFs activates caspase -8 which would suggest apoptotic induction through effects on the plasma membrane that would mimic a mitochondrial independent type I apoptotic pathway. Additional studies are required to examine factors associated with plasma membrane that activates caspase -8 to support this hypothesis.

The initiator caspases are responsible for activating executioner caspases that results in cellular destruction defining the characteristic features of apoptosis. NsPEFs were shown to initiate the activation of executioner caspases -3, -6 and -7 in an electric field dependent manner corresponding with fluorescent microscopy images strengthening executioner caspases association in apoptotic induction. Data suggest that nsPEFs initiate activation of executioner caspases either through direct cleavage of executioner caspases, direct cleavage of initiator caspases leading to executioner caspase activation or through an extrinsic type I apoptotic pathway that elicit effects through the plasma membrane. As demonstrated in Chapter II, the plasma membrane markers that were used here including propidium iodide and Annexin-V did not indicate any direct effects on the plasma membrane. However, Yo-Pro-1, which is similar in size to propidium iodide and has been reported to be specific for apoptotic cells, did cross the plasma membrane indicating selective uptake of Yo-Pro-1 but propidium iodide uptake.

The evasion of cell death via mutations in apoptosis mechanisms is a major step toward malignant transformation and therapeutic resistances. Furthermore, inactivation of apoptosis mechanisms is a hallmark of cancer and an obligate aspect for the malignant transformation of normal cells. Apoptosis pathways mediated by mitochondria exhibit many of the mutations that occur in melanoma. These mitochondria mediated pathways

are commonly activated by chemotherapeutic agents, which often develop resistances. The application of short treatment time for nsPEFs (3 milliseconds) and the rapid activation of caspases (tens of minutes) prove advantageous in apoptosis induction without utilizing mitochondria dependent apoptotic pathway that many chemotherapeutics uses in the initiation of cell death.

In summary, the data revealed that nsPEFs induce a mitochondrial independent apoptosis that involves activation of initiator caspases -8 and unexpectedly caspase -9. This is likely through direct cleavage or an unknown mechanism that is independent of apoptosome formation. In addition the activation of executioner caspases -3, -6 and -7 maybe responsible for mediating morphological changes indicative of apoptosis such as plasma membrane blebbing and F-actin cytoskeleton disruption. Overall, nsPEFs initiate cell death through a mitochondrial independent apoptotic pathway that may involve the plasma membrane.

CHAPTER IV

CONCLUSION

The mechanism of apoptosis induction in B16F10 melanoma cells differs between treatment with the anticancer drug, phloretin and nsPEFs. Phloretin mediates both a mitochondrial dependent intrinsic and type II apoptotic pathway. However, nsPEF-induced melanoma cell death is mediated by a caspase-dependent, calcium and mitochondrial-independent apoptotic induction. NsPEF-induced apoptosis did not reveal effects through the plasma membrane such as phosphatidylserine externalization. The most intense nsPEF conditions tested (ten 300ns pulses at 60kV/cm) did not induce a direct electric field effect on membrane permeability measured by propidium iodide uptake. Changes in membrane permeability were time-dependent biological response to nsPEFs. NsPEFs induce cell death in B16F10 melanoma cells *in vitro* that differs from the conventional intrinsic and extrinsic apoptotic pathway. However nsPEFs induce caspase-dependent apoptosis through pathways that are generally not used by chemotherapeutic agents and ionizing radiation, which often require mitochondria-mediated mechanisms. Many cancer causing mutations in melanoma occur in the mitochondria pathways but can occur in plasma-mediated apoptosis mechanisms. Regardless of any mutations that may occur in B16F10 melanoma cells, nsPEFs can rapidly bypass mitochondria-dependent apoptosis and cause caspase-dependent cell death. This rapid induction of apoptosis and the short treatment time (milliseconds to seconds) for nsPEFs are less likely to induce resistant cancers. Unlike chemotherapy nsPEFs do not have systemic side effects and unlike surgery it is minimally invasive and

leaves no scars. Therefore, the therapeutic application of nsPEFs can provide an inexpensive, new, novel and effective method to the arsenal of cancer treatment strategies.

CHAPTER V

FUTURE STUDIES

The experimental data proves that nsPEFs induce cell death via a mitochondrial independent apoptotic pathway suggesting an extrinsic type I pathway that involves the plasma membrane. In general, the extrinsic type I pathway becomes activated through activation of death receptors and death ligands. Death domain receptors recruit death adaptor proteins (FADD, TRADD), pro-caspase-8 and forms DISC that leads to caspase -8 activation followed by caspase -3. This pathway can be inhibited by c-FLIP which blocks caspase -8 thus preventing caspase -8 recruitment to DISC, resulting in inhibition of apoptosis.

In order to conclusively identify this pathway would require further investigation of proteins associated with the plasma membrane. One approach would be to transform a c-FLIP plasmid into bacteria and then transfect it into B16F10 melanoma cells. This would be followed by examination of caspase -8 activity when nsPEFs are administered to indicate the involvement of the extrinsic type I pathway. The anticipated results should demonstrate a significant reduction in the percentage of cells that express caspase -8 in comparison to cell without c-FLIP plasmid.

Another approach would be to inhibit caspase -8 and examine the percentage of cells that express executioner caspases -3, -6 and -7 in comparison to cells without caspase -8 inhibitor. The rapid activation of nsPEF-induced caspase activity suggests that transcription dependent events are unlikely involved caspase activation. Information could be gained by to investigating changes in the levels of proteins that might be

transcription dependent, such as Bcl-2 family members and survival proteins. While these events may occur in steps post-caspase activation, that nsPEFs induce processes causing caspase activation remains to be identified.

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